

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

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15.9.00

CTM ~~33~~

WRITTEN OPINION

COMPUTER

NOTED

(PCT Rule 66)

CTM

Date of mailing
(day/month/year)

15.06.2000

Applicant's or agent's file reference

P005002WOCTH

REPLY DUE

within 3 month(s)
from the above date of mailing

International application No

PCT/IB99/01465

International filing date (day/month/year)

11/08/1999

Priority date (day/month/year)

11/08/1998

International Patent Classification (IPC) or both national classification and IPC

C12N15/54

Applicant

DANISCO A/S et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☒ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability: citations and explanations supporting such statement
- VI ☒ Certain document cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is **11/12/2000**.

Name and mailing address of the international preliminary examining authority



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WRITTEN OPINION

International application No. PCT/IB99/01465

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

Description, pages:

1-73 as originally filed

Claims, No.:

1-27 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description. pages:
- ☐ the claims. Nos.:
- ☐ the drawings. sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
- ☐ copy of the earlier application whose priority has been claimed.
 - ☐ translation of the earlier application whose priority has been claimed.
2. ☒ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 19, 27.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 19, 27 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-8, 10-21, 23-27
Inventive step (IS)	Claims	1-27
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item II

Priority

Priority is considered **not** to be **valid**. The priority document pertains to components which are "detoxifiable", whereas the claims and the description of the present application relate to components which are "utilisable" (e.g. pages 5/6 of the priority document and page 6 and claims 1-3 of the present application). The scope of the term "utilisable" is broader than that of the term "detoxifiable" as it does not only comprise "detoxifiable" but also "metabolisable" and in addition is open to be interpreted in some other ways.

The P-documents cited in the search report (see section VI) therefore are relevant for assessing novelty of some of the claims of the present application. The documents cited under VI seem to be relevant for the novelty of claims 4-19 and 27 of the present application.

Although Figures 3 and 4 of the present application were not part of the priority document, their subject-matter formed part of the written disclosure of the priority document. Therefore Figures 3 and 4 of the present application are covered by the priority rights.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 19 and 27 were only partly examined. For details see section VIII of this communication.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

- D1: SCHÜMPERLI, D., ET AL.: 'Efficient expression of Escherichia coli galactokinase gene in mammalian cells' PROC. NATL. ACAD. SCI., vol. 79, 1982, pages 257-261

- D2: WO 93 05163 A (DANISCO) 18 March 1993 (1993-03-18)
- D3: DÖRMANN ET AL: 'The role of UDP-glucose epimerase in carbohydrate metabolism of Arabidopsis' PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 13, no. 5, 1 March 1998 (1998-03-01), pages 641-652
- D4: WO 94 20627 A (SANDOZ AG ; SANDOZ AG (DE); SANDOZ LTD (CH); BOJSEN KIRSTEN (DK); D) 15 September 1994 (1994-09-15)
- D5: EP-A-0 235 112 (SMITHKLINE BECKMAN CORP) 2 September 1987 (1987-09-02)
- D6: DÖRMANN ET AL: 'Functional expression of Uridine 5'diphospho-glucose 4-epimerase (EC 5.1.3.2) from Arabidopsis thaliana in Saccharomyces cerevisiae and Escherichia coli' ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, US, NEW YORK, US, XP002076759 ISSN: 0003-9861
- D7: JOERSBO, M., ET AL.: 'Analysis of mannose selection used for transformation of sugar beet' MOLECULAR BREEDING, vol. 4, April 1998 (1998-04), pages 111-117
- D8: WO 96 31612 A (SEMINIS VEGETABLES ; TRULSON ANNA JULIA (US); GREEN CHARLES EDWARD) 10 October 1996 (1996-10-10)
- D9: JOHANSEN I E: 'INTRON INSERTION FACILITATES AMPLIFICATION OF CLONED VIRUS CDNA IN ESCHERICHIA COLI WHILE BIOLOGICAL ACTIVITY IS REESTABLISHED AFTER TRANSCRIPTION IN VIVO' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, vol. 93, no. 22, 29 October 1996 (1996-10-29), pages 12400-12405

The present application deals with a selection method using the ability of plants to grow on D-galactose as a carbon source as a distinguishing feature.

1. Novelty (Article 33(2) PCT):

The following claims are considered not to be novel:

Claims 1-8, 10-18, 20, 21, 23-26; 19 and 27 (see also sections III and VIII)

- 1.1 Document D1 discloses the insertion of the E. coli galactokinase gene into a plasmid. The plasmid is then used for the transfection of hamster cells deficient in

galactokinase activity. The complementation of the defect leads to the selective survival of the transformed cells which selectively grow on galactose as sole carbon source in the medium.

According to their present wording, claims 1-8, 10, 14, 20, 21, and 23-27 are therefore anticipated by D1. The term "toxic" (e.g. in claim 25) was interpreted according to the definition given in the application on page 18.

- 1.2 Document D2 discloses a selection method making use of the introduction of a nucleotide sequence (or a co-introduced nucleotide sequence), wherein the product of the introduced sequence induces or increases the positive effect of a compound or nutrient. Of particular relevance are claims 1-7, 21 and 24 of D2 in which the use of galactose as a compound and of UDP-galactose epimerase as an enzyme useful for the selection of transformed plant cells are explicitly mentioned. As acknowledged by the applicant on page 22, l. 30 and 31 of the present application, the toxicity of galactose for most plants was known for decades.

In view of D2 claims 1-8, 10-21, and 23-27 cannot be considered novel.

- 1.3 Documents D3 to D8 are relevant for the novelty of various claims of the present application. These documents represent the state of the art concerning the enzymes of galactose metabolism, especially in plants, and the use of sugars such as mannose or xylose as selective markers. However, their subject-matter does not contribute to any objections going beyond those already mentioned in 1.1 and 1.2. For the moment, D3 to D8 therefore will not be discussed in detail.

- 1.4 The documents cited under VI are not referred to in detail as all the claims which are anticipated by these documents are as well anticipated by documents published before the priority date of the present application (see 1.1-1.3).

2. Inventive step (Article 33(3) PCT):

Claims 9 and 22 which were not objected to under Article 33(2) PCT are considered not to involve any inventive activity as required by Article 33(3) PCT.

- 2.1 Although the documents cited under V. 1. disclose various ways of selecting

transformed plant cells without using antibiotics or herbicide resistance genes as selective markers, none of them directly mentions a kit comprising the respective construct and the medium. However, it is clear that both are necessary to carry out the selection procedure. As a consequence, claim 9 of the present application does not involve any inventive activity. In contrast, it might also be concluded that the features are implicitly comprised in the prior art and therefore not novel.

- 2.2 Claim 22 relates to a prokaryote comprising a nucleic acid which is prevented from being expressed by the introduction of at least one intron. Document D9 discloses that the introduction of an intron facilitates the expression of cloned cDNA in *E. coli*. D9 generally teaches that intron insertion facilitates manipulation and amplification of cloned DNA fragments (see abstract). The introduction of an intron in a gene involved in the galactose metabolism can therefore not be considered inventive. In addition, at the time the present application was filed, several human genes involved in the galactose metabolism were already cloned. The genomic sequences of these genes contain introns and therefore might have been used for the purpose indicated without further alteration.
- 2.3 In case that novelty of some of the claims discussed in V, 1. should be restored, the subject-matter of claims 1-8, 10-22, and 23 to 27 will be considered not inventive in view of documents D2 or D4 in combination with D3. Documents D2 and D4 both disclose selection methods for transformed plant cells. In D2, galactose and UDP-galactose epimerase are used, and in D4 mannose or xylose are used in combination with mannose-6-phosphate isomerase or xylose isomerase, respectively. Document D3 teaches (p. 642, left column), that mannose and xylose as well as galactose are toxic to plants. On p. 650 it is stated that UDP-glucose epimerase is an interesting transformation marker for genetic engineering of crop plants. At the priority date of the present application it was therefore known how to use enzymes involved in the carbohydrate metabolism as a selective marker and it was also known which compounds are generally useful. The detailed provision of one of these possibilities which were already under discussion does not represent any inventive activity.

**WRITTEN OPINION
SEPARATE SHEET**

International application No. PCT/IB99/01465

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date (day:month:year)	Filing date (day:month:year)	Priority date (valid claim) (day:month:year)
PCT/GB98/00367	13.08.98	05.02.98	07.02.97&02.02.98
PCT/CA99/00056	29.07.99	22.01.99	22.01.98& 23.01.98
PCT/IB98/00886	03.12.98	27.05.98	28.05.97

Re Item VII

Certain defects in the international application

The description of Figure 3 on page 47, l. 23-26 is not in conformity with what can be seen in Fig. 3 (no arrows).

Re Item VIII

Certain observations on the international application

The application lacks clarity with respect to the following:

1. In claims 1-4, 6, and 23-26 reference is made to a "component and/or the derivative thereof and/or the precursor thereof". This formulation either relates to the compounds themselves or to the enzymes metabolising these compounds. However, it is not clear which substances fall within the scope of the claims (Article 6 PCT). At present a skilled person is not able to decide whether a certain substance falls within the scope of the claims or not.
In case that the component is galactose, it is not clear which criteria must be met by a molecule to fall under the definition "derivative". It is also not clear what criteria a precursor of galactose must meet. In the broadest sense, all sugars can be seen as precursors as long as they can be transformed into galactose somehow. In this case the wording is not only unclear but the subject-matter is insufficiently disclosed (Articles 5 and 6 PCT).

The subject-matter even is more obscure in case that the component is a precursor. It is not clear what finally results from this precursor and which substance finally will be utilised by the cells.

2. Claim 19 relates to "a foodstuff or food prepared from...the invention...". This claim cannot be examined in its whole breadth as it probably has not been searched in its whole breadth (see section III).

A foodstuff or food *per se* are known and therefore are not novel (Article 33(2) PCT).

In addition, also starch or oil or any composition prepared from plants of the present application fall within the scope of the claim. It is, however, not possible for the skilled person to identify whether e.g. a certain oil was produced from a plant of the present application or not, as the oil itself does not contain any features which differ from any other oil.

3. Claim 27 is unclear (Article 6 PCT) as it does not contain any technical information of what is being claimed. It is not clear which the "substantial" parts are and what function they should have.

Furthermore it is close at hand that not all subject-matter possibly falling within the scope of this claim has been searched. The claim can therefore be examined only with respect to what has been searched (see section III).

Due to the lack of technical features, several documents anticipate the alleged subject-matter (Article 33(2) PCT, see section V).

4. General statements in the description which imply that the extent of protection may be expanded in some vague and not precisely defined way, such as the "spirit" of an invention (p. 71, l. 19/20 of the application), are objected to (Article 6 PCT and PCT Gazette Section IV, III-4.3a).

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Our Ref: P005002WO CTH LCH LSB

1 August 2000

For the attention of the International Preliminary Examining Authority

Dear Sirs

**International (PCT) Patent Application No PCT/IB99/01465
Danisco A/S**

We refer to the Written Opinion dated 15 June 2000 and file herewith, in triplicate, a replacement set of claims, and a replacement page 47 of the description.

Support for the amendments made to the claims can be found in the description.

In response to the matter raised in Item II, the term "utilisable" has been replaced with "detoxifiable". Support for the term "detoxifiable" can be found, for example, on page 6, lines 19-22 and page 68, line 22 to page 69, line 24.

With regard to claim 18, it is clear from the description that the number of genes which a plant needs in order for it to be able to metabolise galactose will depend on the enzymes which occur naturally in the particular plant. In this respect, the last enzymes (UDP-galactose epimerase) has widespread occurrence in a range of plant species (page 45, lines 22-29). Therefore, it is clear from the description that it is *least likely* that this enzyme will be necessary in order to confer the capacity to metabolise galactose on a plant. It is therefore implicit that in a preferred embodiment, the selectable gene is selected from one or more of the "upstream" enzyme-encoding genes namely galactokinase, UTP-dependent pyrophosphorylase and UDP-glucose-dependent uridylyltransferase.

Support for the amendment made to new claims 19, 20 and 22 can be found throughout the description (see for example page 17, lines 6-9). Support for the amendment to new claim 21 can be found on page 28, lines 27-page 29, line 9.

Our comments on the objections raised by the Examiner in the Written Opinion follow below.

Novelty

D1 describes a specific hamster cell line which is genetically deficient in galactokinase activity and the transformation of such a cell line with the *E.coli* galactokinase gene in restoration of the genetic defects. As observed by the Examiner, the presence of the gene confers on the cells the capacity to grow on galactose as a sole carbon source in the medium. This selection method of transformed cells is classical positive selection, and galactose does not appear to be toxic to non-transformed cells. Equally, galactose is not "detoxifiable" by the transformed cells. The Examiner has mentioned the definition of "toxic" on page 18. From this, it is clear that the component should have an *adverse affect* on the non-transformed cells. Although the non-transformed cells of D1 are unable to grow, this is because they are unable to utilise galactose as a carbon source on a medium which galactose is the *only* available carbon source.

D2 also relates to a positive selection method. Although it is mentioned in D2 (page 11, lines 12-14 and claim 24) that the "inactive compound or nutrient" may be galactose, it is clear from the rest of this reference (for example page 11, lines 28-33) that the inactive compound or nutrient has no direct adverse affect on the non-transformed cells. Hence, we submit that the claims of the present invention are novel over this disclosure.

Inventive Step

The patentability of claim 9 will stand or fall with the patentability of the claims on which it is dependent (i.e. claim 6, 7 and 8). Since the subject matter of these claims is novel and inventive, the subject matter of claim 9 follows suit.

New claim 21 (original claim 22) has been recast as a use form. D9 does not disclose a method for providing a selection means for selecting a genetically transformed cell over a non-transformed cell. Hence, we submit that the subject matter of new claim 21 is both novel and inventive over this reference.

As aforementioned, D2 relates to a positive selection method. D4 also relates to a positive selection using mannose/xylose, whereby the transgenic cells acquire a metabolic advantage. D3 investigates the role of UDP-glucose epimerase in *Arabidopsis* plants by transfecting *Arabidopsis* cells with the appropriate transcript and then growing on a minimal medium where galactose is the only carbon/energy source. Hence, in the selection method described in D3, non-transformed cells are selected against by the fact that they have no available carbon/energy source in the medium compared with transformed cells. The growth inhibition observed with the wild-type control grown on MS medium with 1% (w/v) galactose as opposed to wild-type control grown on MS medium with 1% (w/v) glucose is thus attributable to sub-optimal growth conditions rather than any toxic effect of the galactose.



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In order to ensure that your PCT Chapter II demand is dealt with as promptly as possible you are requested to use the enclosed self-adhesive labels with any correspondence relating to the demand sent to the Munich Office.

One of these labels should be affixed to a prominent place in the upper part of the letter or form etc. which you are filing.

The authors of D3 conclude that the properties of UDP-Glc epimerase make it an interesting transformation marker for genetic engineering of crop plants (page 469 last line to page 650, line 1). The "property" to which they refer is the observation that although all plants grow well on soil, only plants over expressing this enzyme grow well on galactose-only medium. Any selection system based on this property would thus be a positive selection system.

We submit, therefore, that if the skilled person were to combine D2 or D4 with D3 he would not arrive at the negative selection method of the present invention.

Other Matters

An amendment has been made to the description of Figure 3 on page 47 of the description to deal with the matter raised in item VII.

With regard to the matter raised in item VIII.1, the reference to "a derivative" has been removed from claims 1-4, 6 and 23-26. We submit that the meaning of "a precursor" is clear from the description. It is clear from the description (see for example page 14 lines 13-14 and page 18 lines 15-17) that the precursor is metabolically convertible to the component by the transformed cell. Hence, it would be clear to the skilled person that a substance falls within the definition of "a precursor" if it is metabolisable by the cells into a component which is utilisable by the genetically transformed cells, but toxic to the non-transformed cells.

Original claims 19 and 27 have been deleted, so that the objections raised in paragraphs 2 and 3 of item VIII are now moot.

To the extent that the Examiner does not feel able to acknowledge the novelty, inventive step and industrial applicability of all the present claims, or has an added matter objection, and issuance of a further Written Opinion is deemed inappropriate, a personal interview under Rule 66.6 PCT is hereby requested prior to the issue of the International Preliminary Examination Report, preferably in the first instance by telephone.

Please acknowledge safe receipt by stamping and returning one copy of the enclosed Form 1037.

Yours faithfully
for D Young & Co

Dr C T Harding
Authorised Representative

Yet another modification of the galactose selection system relates to the addition of other carbohydrates or other substances to the selection medium which affect the toxicity of galactose. Furthermore, it will also be obvious for those skilled in the art that changes in the employed galactose concentration in the course of the selection can affect selection efficiency.

Thus, the present invention provides a new selection method based on the novel use of galactose and/or a derivative thereof and/or a precursor thereof.

As galactose and the genes encoding the enzymes responsible for the metabolism of galactose are well-characterized, we believe that galactose would be suitable for use as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants), provided that the transgenic cells, tissues, organs, organisms (such as plants) acquire the appropriate genes for metabolising, detoxifying and/or tolerating galactose.

The present invention will now be described only by way of examples in which reference is made to the following Figures:

Figure 3 is presents sequence information; and

Figure 4 is a schematic diagram of a plasmid.

In more detail, Figure 3 is the complete nucleotide sequence of the *E. coli galT* gene with the deduced amino acid sequence shown below the nucleotide sequence. ~~The primers used for the isolation of the gene by PCR are shown with arrows indicating the 5' to 3' direction.~~

In more detail, Figure 4 is a plasmid map of the plant transformation vector pVIC-GalT.

CLAIMS

1. A selection method for selecting from a population of cells one or more selectable genetically transformed cells.

5 wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product
10 and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is ~~utilisable~~ ^{detoxifiable} by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and
15 optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

20 the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component ~~and/or a derivative thereof~~ and/or a precursor thereof and in an amount such that the component is or will be in an amount that is ~~utilisable~~ ^{detoxifiable} by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

25 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is
30 galactose ~~or a derivative thereof~~ or a precursor thereof.

2. A composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is ~~utilisable~~ ^{detoxifiable} by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein the medium comprises the component ~~and/or a derivative thereof~~ and/or a precursor thereof and in an amount such that the component is or will be in an amount that is ~~utilisable~~ ^{detoxifiable} by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

3. A population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

5 optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is ^{detoxifiable} ~~utilisable~~ by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

15 wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

20 wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is galactose ~~or a derivative thereof~~ or a precursor thereof.

4. A selectable genetically transformed cell comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

25 wherein a component is ^{detoxifiable} ~~utilisable~~ by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
5 and

wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is galactose ~~or a derivative thereof~~ or a precursor thereof.

10 5. An organism comprising a selectable genetically transformed cell according to claim 4.

6. A construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

15 the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component is ^{detoxifiable} ~~utilisable~~ by the selectable genetically transformed cells by
20 action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-
25 transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
and

30 wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is galactose ~~or a derivative thereof~~ or a precursor thereof.

7. A vector comprising the construct according to claim 6.
8. A plasmid comprising the construct according to claim 6.
- 5 9. A kit comprising a construct according to claim 6 or a vector according to claim 7 or a plasmid according to claim 8 for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell; and a medium.
- 10 10. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vitro* within a culture.
11. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vivo* within an organism.
- 15 12. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are selectable genetically transformed plant cell/cells.
13. The invention according to any one of the preceding claims wherein an additional nucleotide sequence is present and wherein the additional nucleotide sequence codes for a
20 nucleotide sequence of interest.
14. The invention according to any one of the preceding claims wherein the component is present in the medium.
- 25 15. The invention according to any one of claims 1 to 13 wherein the component is prepared *in situ* in the cell from a precursor that was present in the medium.
16. A plant prepared from or comprising the invention according to any one of the preceding claims, preferably wherein the plant is capable of providing a feed, foodstuff to
30 humans or animals.

17. A plant according to claim 14 wherein the plant is any one of rape seed, potato or maize.

18. A plant comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10) ^{and/or} UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), ~~UDP-galactose epimerase (EC 5.1.3.2)~~

~~19. A foodstuff or food prepared from or comprising the invention according to any one of the preceding claims.~~

19 ~~20~~. Use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed ^{plant} cell over a non-transformed ^{plant} cell.

20 ~~21~~. Use of any one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed ^{plant} cell over a non-transformed ^{plant} cell.

21 ~~22~~. ^{Use of a} ~~prokaryote~~ comprising a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) ^{and/or} UDP-galactose epimerase (EC 5.1.3.2); wherein the nucleotide sequence comprises at least one intron which inactivates the nucleotide sequence or the expression product thereof in the prokaryote, *for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.*

22 ~~23~~. Use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or a derivative thereof, as a selection means for selecting a genetically transformed ^{plant} cell over a non-transformed ^{plant} cell.

23 ~~24~~ A selection system that uses a component or a precursor therefor or a derivative thereof for selecting at least one genetically transformed cell from a population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but
 5 wherein the component toxic to non-transformed cells, wherein the component ~~and/or the derivative thereof~~ and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

~~25. A selection system that uses a component or a precursor therefor or a derivative thereof~~ for selecting at least one genetically transformed cell from a population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but
 10 ~~wherein the component toxic to non-transformed cells.~~

15 ²⁴ ~~26~~ A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first
 20 expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

25 the method comprising the step of preparing the population of cells by transforming some or all of the cells in an initial population of cells containing non-transformed cells with a heterologous nucleotide sequence so as to form the population of cells containing one or more selectable genetically transformed cells; and

selecting at least one of the selectable genetically transformed cells;

30 wherein the heterologous nucleotide sequence is any one or more of the first expressable nucleotide sequence, the optional second expressable nucleotide sequence or the optional third expressable nucleotide sequence;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose~~or a derivative thereof~~ or a precursor thereof.

- 5 ~~27. A selection method or transformed cell, tissue, organ, or organism (or part thereof) substantially as described herein/~~

5 Yet another modification of the galactose selection system relates to the addition of other carbohydrates or other substances to the selection medium which affect the toxicity of galactose. Furthermore, it will also be obvious for those skilled in the art that changes in the employed galactose concentration in the course of the selection can affect selection efficiency.

10

Thus, the present invention provides a new selection method based on the novel use of galactose and/or a derivative thereof and/or a precursor thereof.

15

As galactose and the genes encoding the enzymes responsible for the metabolism of galactose are well-characterized, we believe that galactose would be suitable for use as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants), provided that the transgenic cells, tissues, organs, organisms (such as plants) acquire the appropriate genes for metabolising, detoxifying and/or tolerating galactose.

20

The present invention will now be described only by way of examples in which reference is made to the following Figures:

Figure 3 presents sequence information; and

25

Figure 4 is a schematic diagram of a plasmid.

In more detail, Figure 3 is the complete nucleotide sequence of the *E. coli galT* gene with the deduced amino acid sequence shown below the nucleotide sequence.

30

In more detail, Figure 4 is a plasmid map of the plant transformation vector pVIC-GalT.

5 **CLAIMS**

1. A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed
10 cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second
15 expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is detoxifiable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide
20 sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

25 wherein the medium comprises the component and/or a precursor thereof and in an amount such that the component is or will be in an amount that is detoxifiable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second
30 expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the precursor thereof is galactose or a precursor thereof.

5

2. A composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

10 optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is detoxifiable by the selectable genetically transformed
15 cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-
20 transformed cells;

wherein the medium comprises the component and/or a precursor thereof and in an amount such that the component is or will be in an amount that is detoxifiable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

25 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the precursor thereof is galactose or a
30 derivative thereof or a precursor thereof.

- 5 3. A population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

 wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

 optionally wherein each of the selectable genetically transformed cells
10 comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

 wherein a component is detoxifiable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression
15 product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

 wherein the component can be present in an amount that is toxic to the non-transformed cells;

20 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

 wherein the component and/or the precursor thereof is galactose or a precursor
25 thereof.

4. A selectable genetically transformed cell comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third
30 expressable nucleotide sequence encoding a third expression product;

 wherein a component is detoxifiable by the selectable genetically transformed cells by action of the the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional
35 third expressable nucleotide sequence or the optional third expression product;

5 wherein the component can be present in an amount that is toxic to the non-transformed cells;

 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

10 wherein the component and/or the precursor thereof is galactose or a precursor thereof.

5. An organism comprising a selectable genetically transformed cell according to claim 4.

6. A construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

 the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

 wherein a component is detoxifiable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

 wherein the component can be present in an amount that is toxic to the non-transformed cells;

 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

 wherein the component and/or the precursor thereof is galactose or a precursor thereof.

- 5 7. A vector comprising the construct according to claim 6.
8. A plasmid comprising the construct according to claim 6.
9. A kit comprising a construct according to claim 6 or a vector according to
10 claim 7 or a plasmid according to claim 8 for genetically transforming a non-
transformed cell to produce a selectable genetically transformed cell; and a medium.
10. The invention according to any one of the preceding claims wherein the
selectable genetically transformed cell/cells is/are *in vitro* within a culture.
- 15 11. The invention according to any one of the preceding claims wherein the
selectable genetically transformed cell/cells is/are *in vivo* within an organism.
12. The invention according to any one of the preceding claims wherein the
20 selectable genetically transformed cell/cells is/are selectable genetically transformed
plant cell/cells.
13. The invention according to any one of the preceding claims wherein an
additional nucleotide sequence is present and wherein the additional nucleotide
25 sequence codes for a nucleotide sequence of interest.
14. The invention according to any one of the preceding claims wherein the
component is present in the medium.
- 30 15. The invention according to any one of claims 1 to 13 wherein the component
is prepared *in situ* in the cell from a precursor that was present in the medium.
16. A plant prepared from or comprising the invention according to any one of the
preceding claims, preferably wherein the plant is capable of providing a feed,
35 foodstuff to humans or animals.

5 17. A plant according to claim 14 wherein the plant is any one of rape seed, potato or maize.

18. A plant comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-
10 dependent pyrophosphorylase (EC 2.7.7.10) and/or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12).

19. Use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC
15 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed plant cell over a non-transformed plant cell.

20. Use of any one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for
20 providing a selection means for selecting a genetically transformed plant cell over a non-transformed plant cell.

21. Use of a prokaryote comprising a nucleotide sequence coding for
25 galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) and/or UDP-galactose epimerase (EC 5.1.3.2); wherein the nucleotide sequence comprises at least one intron which inactivates the nucleotide sequence or the expression product thereof in the prokaryote, for providing a selection means for selecting a genetically transformed
30 cell over a non-transformed cell.

22. Use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or a derivative thereof, as a selection means for selecting a genetically transformed plant cell over a non-transformed plant cell.

35

23. A selection system that uses a component or a precursor therefor or a derivative thereof for selecting at least one genetically transformed cell from a

5 population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but wherein the component toxic to non-transformed cells, wherein the component and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

10

24. A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

15

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

20

the method comprising the step of preparing the population of cells by transforming some or all of the cells in an initial population of cells containing non-transformed cells with a heterologous nucleotide sequence so as to form the population of cells containing one or more selectable genetically transformed cells;

25

and

selecting at least one of the selectable genetically transformed cells;

wherein the heterologous nucleotide sequence is any one or more of the first expressable nucleotide sequence, the optional second expressable nucleotide sequence or the optional third expressable nucleotide sequence;

30

- 5 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof.

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DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA

Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference

P005002WO CTH

International application No.

International filing date (day/month/year)

(Earliest) Priority date (day/month/year)

PCT/IB99/01465

11 Aug 1999

11 Aug 1998

Title of invention

SELECTION METHOD

Box No. II APPLICANT(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Telephone No.:

Danisco A/S
Langebrogade 1
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Facsimile No.:

Teleprinter No.:

State (that is, country) of nationality: Denmark

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State (that is, country) of residence: Denmark

☒ Further applicants are indicated on a continuation sheet

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet is not to be included in the demand.

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)

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State (that is, country) of nationality:

State (that is, country) of residence:

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)

State (that is, country) of nationality:

State (that is, country) of residence:

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)

State (that is, country) of nationality:

State (that is, country) of residence:

Further applicants are indicated on another continuation sheet

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCEThe following person is ☒ agent common representativeand ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked

is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *Family name followed by given name, for a legal entity, full official designation
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Address for Correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION**Statement concerning amendments: ***

1. The applicant wishes the international preliminary examination to start on the basis of:

☒ the international application as originally filed

the description

as originally filed

as amended under Article 34

the claims

as originally filed

as amended under Article 19 (together with any accompanying statement)

as amended under Article 34

the drawings

as originally filed

as amended under Article 34

2. The applicant wishes any amendment to the claims under Article 19 to be considered as reversed

3. The applicant wishes the start of the international preliminary examination to be postponed until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)).
- (This check-box may be marked only where the time limit under Article 19 has not yet expired).*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination:

English

☒ which is the language in which the international application was filed

which is the language of a translation furnished for the purposes of international search.

which is the language of publication of the international application.

which is the language of translation (to be) furnished for the purposes of international preliminary examination

Box No. V ELECTION OF STATES

The applicant hereby elects all eligible States (that is, all States which have been designated and which are bound by Chapter II of the PCT)

excluding the following States which the applicant wishes not to elect:

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

- | | | | |
|---|---|-------|--------|
| 1 | translation of international application | _____ | sheets |
| 2 | amendments under Article 34 | _____ | sheets |
| 3 | copy (or, where required, translation) of amendments under Article 19 | _____ | sheets |
| 4 | copy (or, where required, translation) of statement under Article 19 | _____ | sheets |
| 5 | letter | _____ | sheets |
| 6 | other (specify) | _____ | sheets |

For International Preliminary
Examining Authority use only

received

not received

The demand is also accompanied by the item(s) marked below:

- | | | | |
|----|---|----|--|
| 1. | <input checked="" type="checkbox"/> fee calculation sheet | 4. | <input type="checkbox"/> statement explaining lack of signature |
| 2. | <input type="checkbox"/> separate signed power of attorney | 5. | <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form |
| 3. | <input type="checkbox"/> copy of general power of attorney; reference number, if any: | 6. | <input checked="" type="checkbox"/> other (specify) Letter |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand)

C T HARDING - Authorised Agent

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply

The applicant has been informed accordingly.

4. The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5

5. Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82

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Demand received from IPEA on

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No PCT/IB99/01465	For international Preliminary Examining Authority use only	
Applicant's or agent's file reference P005002WO CTH	Date stamp of the IPEA	
Applicant <div style="text-align: center; margin-top: 10px;">DANISCO A/S</div>		
Calculation of prescribed fees		
1. Preliminary examination fee	EUR 1,533	P
2. Handling fee (<i>Applicants from certain states are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.</i>)	EUR 147	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	EUR 1,680	
	TOTAL	
Mode of Payment		
<input checked="" type="checkbox"/> authorization to charge deposit account with the IPEA (see below)	cash	
<input type="checkbox"/> cheque	revenue stamps	
<input type="checkbox"/> postal money order	coupons	
<input type="checkbox"/> bank draft	other (specify):	
Deposit Account Authorization <i>(this mode of payment may not be available at all IPEAs)</i>		
The IPEA/ EPO <input checked="" type="checkbox"/> is hereby authorized to charge the total fees indicated above to my deposit account.		
<input checked="" type="checkbox"/> <i>(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)</i> is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.		
28050042	14 Feb 2000	
Deposit Account Number	Date (day/month/year)	Signature Charles Harding

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/54, 15/61, 15/82, 5/10, 1/21, A01H 5/00	A2	(11) International Publication Number: WO 00/09705 (43) International Publication Date: 24 February 2000 (24.02.00)
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<p>(54) Title: SELECTION METHOD</p> <pre> ACGCGAGAAACACCAATGACCAATTTAATCCGTTGATCATCCATCCGCGGTACAAACCGGTCACCGGGCAATGGATTCTGGTTTCACCGACCGCG 180 PstI: N T Q F M P V D K P H E R Y M P L T G O M I L V S P H R 28 CTAAGCGCCCTCGGAGGCGCGGAGGAAACCGGAGGTTTACCTGCGGAGGATCCAGATTCTCTCTGCGCAAGTAATGCGGGTGAC 200 A K R P W Q G A G E T P A K Q V L P A H D P D C F L C A G N V R V T 62 AGGCGATAAAACCGGATTACCGGACTTACGTTTCACTAATGACTTTGCGGCTTGTATGTCGACGCGGAGATCGCGCAGAAATCAGCATCG 380 G D K N P D Y T G T Y V F T H D P A A L N S D T P D A P E S N D P 95 CTGATGCTTTCGAGAGCGCGCGCGACCGCGGATGATCTGCTTTTCACGAGATCAGTAAGCGCTGCGAGAGCTCAGCGTTCGACGATTGACGG 400 L M R C O S A R G T S R V I C F S P D R S K T L P E L S V A A L T 128 AATCCTCAAAACCTGGCAGGACAAACCGCGGAACTGGGAAACGTAACCTAGGTCAGGTTTGAAGAAAGAGCGCGCGGATGGCTGCTCTAA 560 S I V K T N O E Q T A E L G E T Y P M V Q V P E N K G A P H G C S M 162 CCGCGATCGGACGCTCAGATTGGGCAATAGCTTCTCGCTAACGAGCTGAGCGGAGCGCGCTGCAAAAGAAATATTTCGCGAAGCAAAATCA 600 P H P H G Q I M A N S F L P R E A E R E D R L O K E Y P A K Q K S 195 CCAATGCTGGTGGATTGTTGACCGGAGCTGCGAGCGGATGCGCTACCTGTTGTAACCGAAGCACTGGTTAGCGCTGCTGCTTACTGGCTGCT 700 P M L V D Y V Q R E L A D G S R T V V E T E K N L A V V P Y M A A 228 GGCGTTGGAACGCTACTGCTGCGGAAAGCGGACGTTTACGAGTACGAGTTTGAAGGAGCGGAGCGGATGCGGCTGCGCTTGAAGAGCT 800 M F F E T L L L P K A N V L R I T D L T D A Q R S D L A L A L E K L 262 CAGCGAGCTGTTATGACAACTCTTCCAGTCTCTCTCCCTACTCTATGGCTGCGAGCGGCGGCAATTAATGGGAGAGAAATCAACACTGCGAGCTG 900 T S R Y D N L F Q C S P P Y S H G M G A P P H G E E R Q H M Q L 295 CAGCGGACCTTTATCCGCTCTGCTGCGCTCGCGCACGCTAGCTAATTTATGGTTGTTATGAATGCTGCGAGAGCGGAGCGGAGCTGCGGCGAG 1080 N A K F Y P P L L R E A T V R K F N V G Y E N L A E T Q R D L T A 328 AAGAGGAGGAGCGCTTTCGCGGAGTACGATATCCATTTTCGGAATCCGAGTGTAACTGAGT 1069 S Q A A E R L R A V S D I N F R E S G V - S e l I 348 </pre>
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(57) Abstract

A selection method for selecting from a population of cells one or more selectable genetically transformed cells is described. The population of cells comprises selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressible nucleotide sequence encoding a first expression product; and optionally a second expressible nucleotide sequence encoding a second expression product and/or a third expressible nucleotide sequence encoding a third expression product. A component is utilisable by the selectable genetically transformed cells by action of the first expressible nucleotide sequence or the first expression product and optionally by action of the optional second expressible nucleotide sequence or the optional second expression product and/or by action of the optional third expressible nucleotide sequence or the optional third expression product. The component can be present in an amount that is toxic to the non-transformed cells.

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SELECTION METHOD

The present invention relates to a selection method.

- 5 The present invention also relates to an enzyme and a nucleotide sequence coding for same that are useful in a selection method.

In particular, the present invention relates to a method for the selection (e.g. identification and/or separation) of genetically transformed cells and compounds and
10 genetic material for use in the method.

It is well known that when a nucleotide sequence of interest ("NOI") is to be introduced into a population of cells by transformation, only a certain number of the cells are successfully transformed, i.e., only a certain number of the cells receive the NOI. It is
15 then necessary to identify the genetically transformed cells so that these cells may be separated from the non-transformed cells in the population. For the production of transgenic plants etc., this often requires the use of a selection system that allows the regeneration and growth of the transformed (or transgenic) cells. As these transformed cells frequently constitute a minor fraction of the treated cells, compared to the majority
20 of cells which remain untransformed, so the selection system has to be able to be effective in selecting out the transformed cells.

A common technique for a selection method includes introducing transformed cells and non-transformed cells into a medium that comprises a substance which the transformed
25 cells are able to tolerate. In that medium the transformed cells are able to survive and grow, while the non-transformed cells are prone to growth inhibition and, in some cases, are killed.

Thus, to date, the general strategy has been to introduce a selectable gene along with the
30 NOI(s), and then allowing the transformed cells to survive on selective media while the non-transformed cells are killed (Bowen 1993).

Typically, if a population of plant cells has been subjected to genetic transformation, selection of the transformed cells typically takes place using a selection gene which codes for antibiotic resistance or herbicide resistance. The selection gene is coupled to or co-introduced with the NOI to be incorporated into the plant in question, so that both of the two sequences are incorporated into some or all of the population of cells.

As not all of the cells may have been transformed, the cells are then cultivated on or in a medium containing the respective antibiotic or herbicide to which the genetically transformed cells are resistant by virtue of the selection gene. In this medium, the transformed cells are able to grow and thus be identified out of the total cell population, since the non-transformed cells - which do not contain the antibiotic or herbicide resistance gene in question - have an inhibited growth or even are killed.

So far, the most widely used selectable gene is the neomycin phosphotransferase II (NPTII) gene (Fraley *et al.* 1986) which confers resistance to the aminoglycoside antibiotics kanamycin, neomycin and G-418 (Bevan *et al.* 1983). A number of other selective systems has been developed based on resistance to bleomycin (Hille *et al.* 1986), bromoxynil (Stalker *et al.* 1988), chloramphenicol (Fraley *et al.* 1983), 2,4-dichlorophenoxy-acetic acid (Streber and Willmitzer 1989), glyphosate (Shah *et al.* 1986), hygromycin (Waldron *et al.* 1985) or phosphinothricin (De Block *et al.* 1987).

The selection methods which rely on the use of antibiotics or herbicides suffer from a number of disadvantages. For example, there is concern amongst some people, such as environmental groups and governmental authorities, as to whether it is environmentally safe to incorporate genes coding for antibiotic resistance and/or herbicide resistance into plants and micro-organisms. This concern is of particular significance for food plants and for micro-organisms which are not designed and/or intended to be used in a closed environment (e.g. micro-organisms for use in agriculture), and also for micro-organisms which are designed for use in a closed environment but which may be released from the closed environment.

While such ecological concerns may prove unfounded, as suggested by Flavell *et al.* (1992), they may nevertheless lead to governmental restrictions on the use of antibiotic resistance genes in transgenic plants, and it is therefore desirable to develop new selection methods which are independent of such genes.

In addition, in some or many cases, the corresponding antibiotic or herbicide resistance genes may not be relevant to the desired transgenic trait. Also, they may be undesirable in the final product (Yoder and Goldsbrough 1994).

Thus, the use and subsequent release of selectable genes such as antibiotic resistance genes into the environment has been the target of concern among environmental authorities.

Therefore, new selection systems for the production of transgenic plants without any herbicide or antibiotic resistance genes have been developed. By way of example, these new selection systems include three selection systems based on the concept of 'positive selection' wherein transgenic cells acquire a gene which confers a metabolic advantage to those cells whilst non-transgenic cells starved rather than killed. Some of these selection systems have been reviewed by Joersbo (Joersbo 1997).

One of these positive selection methods relates to cytokinins which must be added to obtain optimal shoot regeneration. By adding cytokinin as an inactive glucuronide derivative and using a β -glucuronidase gene as selectable gene, cells which have acquired this gene by transformation are able to convert the cytokinin glucuronide to active cytokinin while untransformed cells are arrested in development (Joersbo and Okkels 1996; Okkels *et al.* 1997).

Two other selection systems employ, as selectable agents, the carbohydrates mannose and xylose, which are not metabolised by a number of plant species (Bojsen *et al.* 1994). By substituting the normally employed carbohydrate with one of these compounds, cells transformed with a gene encoding an enzyme capable of converting it to a metabolisable

isomer are favoured in growth while the non-transgenic cells are starved. Mannose is initially phosphorylated to mannose-6-phosphate by hexokinase present in plant cells but this compound is not metabolised any further in many plant species. Cells transformed with a phosphomannose isomerase gene are able to convert mannose-6-phosphate to the readily metabolised fructose-6-phosphate, giving these cells a metabolic advantage (Joersbo *et al.* 1998). Xylose can be converted to xylulose by xylose isomerase which functions as the selectable marker in this system (Haldrup 1996).

Another selection system that is not dependent on the use of herbicide and antibiotic resistance genes is described in PCT/GB98/00367. In the general method of PCT/GB98/00367, selectable transformed cells are selected from a population of cells which comprises the selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence and optionally a second expressable nucleotide sequence. In the selection method, a component or a metabolic derivative thereof when present in a low concentration in a medium is a nutrient for both the selectable genetically transformed cells and the non-transformed cells. In the selection method, the component or the metabolic derivative thereof when present in a high concentration in a medium is toxic to the non-transformed cells. The first nucleotide sequence codes for a gene product capable of converting the component or the metabolic derivative thereof when present in a high concentration in a medium to a nutrient for the selectable genetically transformed cells. The selection method comprises the step of introducing the population of cells to a medium, wherein the medium optionally comprises a high concentration of the component or the metabolic derivative thereof. In the selection method, the component or the metabolic derivative thereof is a source of both carbohydrate and nitrogen for the selectable genetically transformed cells. Alternatively, in the selection method if a portion of the component serves as a metabolic substrate and is metabolically converted to a derivatised substrate, then that derivatised substrate is capable of providing an allosteric effect on the gene product. In one preferred aspect, the selection method relies on the use of glucosamine.

Despite the advent of selection methods that do not necessarily rely on the use of antibiotic or herbicide resistance genes, it is still desirable to develop new methods for selecting genetically transformed cells or organisms (or parts thereof) comprising such.

5 According to a first aspect of the present invention there is provided:

a selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

10 wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third
15 expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable
20 nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and/or a derivative thereof and/or
25 a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an
30 enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

For convenience, we sometimes refer to the selection method of the present invention as being the "galactose selection method". However, it is to be understood that the method of the present invention is not necessarily limited to galactose as being the component or the precursor of the component that enables workers to select transformed cells over non-transformed cells. However, and as will become apparent, in a preferred embodiment galactose is a preferred component or precursor for the component and/or galactose-1-phosphate is a preferred component or derivative thereof.

The component may be any suitable chemical compound, product, molecule etc.

The term "utilisable" as used herein with reference to the present invention (e.g. "the component is utilisable by the selectable genetically transformed cells") means that the component can be processed in a non-adverse or non-detrimental fashion by the selectable genetically transformed cells. Thus, the term includes metabolising (such as metabolising in a beneficial manner), detoxifying, etc. For some applications, the term "utilisable" may mean at least "metabolisable". For some applications, the term "utilisable" may mean at least "detoxifiable". Here, the term "detoxifiable" means capable of being converted to one or more derivatives that do not have an adverse effect on the transformed cell. An example of an adverse effect is growth inhibition. These derivatives may or may not accumulate in the cells. In addition, or in the alternative, these derivatives may or may not be fully metabolised by the transformed cell.

The derivative of the component can be any suitable derivative - such as a chemical derivative, a metabolic derivative, etc.

For some applications, preferably the derivative of the component is at least a metabolic derivative.

The precursor of the component can be any suitable precursor - such as a metabolic precursor, etc.

5 For some applications, preferably the precursor of the component is at least a metabolic precursor.

The action of the the first expressable nucleotide sequence or the first expression product and optionally the optional second expressable nucleotide sequence or the optional second expression product and/or the optional third expressable nucleotide sequence or
10 the optional third expression product may be direct or indirect. Here, indirect action may mean that the first expression product or the optional second expression product or the optional third expression product converts a precursor of the component to, for example, the component or even another precursor thereof or a derivative thereof. However, the component that is present or is produced by direct or indirect action is or
15 will be in an amount that is utilisable by the transformed cells but in an amount that is toxic to the non-transformed cells.

According to a second aspect of the present invention there is provided

a composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;
20

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third
25 expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable
30 nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein the medium comprises the component and/or a derivative thereof and/or a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

According to a third aspect of the present invention there is provided

a population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an

enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
and

wherein the component and/or the derivative thereof and/or the precursor thereof
is galactose or a derivative thereof or a precursor thereof.

5

According to a fourth aspect of the present invention there is provided

a selectable genetically transformed cell comprising a first expressable nucleotide
sequence encoding a first expression product; and optionally a second expressable
nucleotide sequence encoding a second expression product and/or a third expressable
10 nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells
by action of the first expressable nucleotide sequence or the first expression product and
optionally by action of the optional second expressable nucleotide sequence or the
optional second expression product and/or by action of the optional third expressable
15 nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-
transformed cells;

wherein each of the first expression product and the optional second expression
product and the optional third expression product is independently selected from an
20 enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
and

wherein the component and/or the derivative thereof and/or the precursor thereof
is galactose or a derivative thereof or a precursor thereof.

25 According to a fifth aspect of the present invention there is provided an organism
comprising a selectable genetically transformed cell according to the present invention.

According to a sixth aspect of the present invention there is provided

a construct for genetically transforming a non-transformed cell to produce a
30 selectable genetically transformed cell;

the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

5 wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

10 wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
15 and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

According to a seventh aspect of the present invention there is provided a vector
20 comprising the construct according to the present invention.

According to an eighth aspect of the present invention there is provided a plasmid comprising the construct according to the present invention.

25 According to a ninth aspect of the present invention there is provided a kit comprising a construct according to the present invention or a vector according to the present invention or a plasmid according to the present invention for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell; and a medium.

According to a tenth aspect of the present invention there is provided a plant or plant cell comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose
5 epimerase (EC 5.1.3.2).

Here the term "heterologous enzymes" includes enzymes that are derived from a different species or even a different cell. The term also includes enzymes (which may or may not be native to the cell) which are expressed by recombinant nucleotide sequences.

10 The term also includes homologous enzymes that have been expressed by homologous coding sequences but when under the control of heterologous promoters. Here the term "heterologous promoters" means promoters that are not naturally associated with the coding sequence in question.

15 According to an eleventh aspect of the present invention there is provided a feed, foodstuff or food prepared from or comprising the aspects of the present invention.

According to a twelfth aspect of the present invention there is provided the use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC
20 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed cell over a non-transformed cell.

According to a thirteenth aspect of the present invention there is provided the use of any
25 one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

According to a fourteenth aspect of the present invention there is provided the use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or derivatives thereof as a selection means for selecting a genetically transformed cell over a non-transformed cell.

According to a fifteenth aspect of the present invention there is provided

a selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

the method comprising the step of preparing the population of cells by transforming some or all of the cells in an initial population of cells containing non-transformed cells with a heterologous nucleotide sequence so as to form the population of cells containing one or more selectable genetically transformed cells; and

selecting at least one of the selectable genetically transformed cells;

wherein the heterologous nucleotide sequence is any one or more of the first expressable nucleotide sequence, the optional second expressable nucleotide sequence or the optional third expressable nucleotide sequence;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof.

Here, the term "heterologous nucleotide sequence" includes nucleotide sequences that are derived from a different species or even a different cell. The term also includes nucleotide sequences (which may or may not be native to the cell) that have been

prepared by use of recombinant DNA techniques. The term also includes native nucleotide sequences but when under the control of heterologous promoters. Here the term "heterologous promoters" means promoters that are not naturally associated with the nucleotide sequence in question.

5

In one preferred aspect, the selectable genetically transformed cell/cells is/are *in vitro* within a culture.

10

In an alternative preferred aspect, the selectable genetically transformed cell/cells is/are *in vivo* within an organism.

Preferably the selectable genetically transformed cell/cells is/are selectable genetically transformed plant cell/cells.

15

Preferably an additional nucleotide sequence is present and wherein the additional nucleotide sequence codes for a nucleotide sequence of interest ("NOI").

Preferably the organism is a plant.

20

Preferably the plant is capable of providing a foodstuff for humans or animals.

In an alternative preferred aspect, the plant is capable of providing a commodity for humans - such as cotton, tobacco etc.

25

Preferably the plant (or part thereof, including cells thereof) is a monocot or a dicot (including legumes).

In a preferred aspect, the plant is any one of rape seed, potato or maize.

30

For some applications, preferably the component is present in the medium.

For some applications, preferably the component is prepared *in situ* in the cell from a precursor that was present in the medium.

5 If the component is prepared *in situ* in the cell from a precursor that was present in the medium, then galactose is a preferred component or precursor for the component and/or a preferred component or derivative thereof is galactose-1-phosphate.

10 More preferably, if the component is prepared *in situ* in the cell from a precursor that was present in the medium, then galactose is a preferred precursor for the component and/or a preferred component is galactose-1-phosphate.

Thus, in each aspect of the present invention, a precursor for the component is preferably metabolically converted to the component by the transformed cell.

15 When the component or the derivative thereof or precursor thereof is present in the medium then preferably the component or the derivative thereof or precursor thereof is present in an amount that does not detrimentally affect a major proportion of the transformed cells.

20 Preferably, when the component or the derivative thereof or precursor thereof is present in the medium then the component or the derivative thereof or precursor thereof is present in an amount that does not detrimentally affect substantially most of the transformed cells.

25 More preferably, when the component or the derivative thereof or precursor thereof is present in the medium then the component or the derivative thereof or precursor thereof is present in an amount that does not detrimentally affect substantially all of the transformed cells.

30 In a further aspect, in some cases the medium need not contain any added quantities of the component or the derivative or precursor thereof according to the present invention.

Preferably, however, the medium contains added quantities of the component or the derivative or precursor thereof according to the present invention.

5 For some applications, if carbohydrates are also present in addition to the component or the derivative thereof or the precursor therefor then preferably the carbohydrates are present in amounts that do not affect substantially the effect of the component or the derivative thereof or the precursor therefor. Typically, said levels of carbohydrate(s) will be low levels.

10 Other aspects of the present invention include:

The use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or any derivative thereof, as a selection means for selecting a genetically transformed
15 cell over a non-transformed cell.

The use of any one or more of a nucleotide sequence coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC
20 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

The use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC
25 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed cell.

In the above aspects, the phrase "selecting a genetically transformed cell over a non-transformed cell" can be alternatively expressed as "selecting a genetically transformed
30 cell from one or more non-transformed cells".

Preferably, any one or more of the first, second or third expressable nucleotide sequence and/or any one or more of the first, second or third expression product (such as any one or more of the enzymes galactokinase, UTP-dependent pyrophosphorylase, UDP-glucose dependent uridylyltransferase and UDP-galactose epimerase and/or nucleotide sequences coding for same) are, for example, obtainable from any one or more of: *E. coli* (CE Vorigas, H-G Lemaire, KS Wilson 1991. Protein Expr. and Pur. 2, 330-338); *Saccharomyces sp.* (RA Darrow, R Rodstrom 1968. Biochemistry 7, 1645-1654; MA Schell, DB Wilson 1977. J. Biol. Chem. 252, 1162-1166); *Streptomyces coelicolor* A3 (BD Wilson, DS Hogness 1966. in Methods in Enzymol. vol. 8, pp. 229-240, Academic Press, San Diego); *Tetrahymena thermophila* (JE Lavine, E Cantlay, CT Roberts, DE Morse 1982. Biochim. Biophys. Acta 717, 76-85); *Clostridium pasterianum* (F Daldal, J Applebaum 1985. J. Mol. Biol. 186, 533-545); *Kluveromyces lactis* (MI Riley, RC Dickson 1984. J. Bacteriol. 158, 705-712); *Vicia faba*; *Petunia sp.*; and mammals eg. Chinese hamster (B Talbot, J-P Thirion 1982. Int. J. Biochem. 14, 719-725), human (WO 96/09374).

According to one aspect of the present invention there is provided a selection system that uses a component or a precursor therefor or a derivative thereof for selecting at least one genetically transformed cell from a population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but wherein the component toxic to non-transformed cells.

According to another aspect of the present invention there is provided a selection system for selecting at least one genetically transformed cell from a population of cells in a medium, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of converting a component or precursor therefor or a derivative thereof, that is present in the medium that provides the component at an amount that is toxic to non-transformed cells but is utilisable (e.g. metabolisable and/or detoxifiable) by the at least one transformed cell.

The term "cells" is intended to refer to any type of cells from which individual genetically transformed cells may be identified and isolated using the method of the invention. Examples of such cells typically include cells of plants that have a commercial worth - such as crops useful for food or feed production. If desired, other plant cells can be transformed. The term "cells" is also meant to encompass protoplasts, i.e. the protoplasm of a cell enclosed in a membrane but without a cell wall. While it is contemplated that the selection method of the present invention may be used for any type of cell, the method has been found to be particularly suitable for the selection of genetically transformed plant cells.

The term "population of cells" refers to any group of cells which has been subjected to genetic transformation and from which it is desired to identify those cells which have been genetically transformed and to isolate the genetically transformed cells from non-genetically transformed cells. The population may, for example, be a tissue, an organ or a portion thereof, a population of individual cells in or on a substrate, such as a culture of plant cells, for example a population of cells in a solution or suspension, or a whole organism, such as an entire plant.

The term "selecting" refers to the process of identifying and/or isolating the genetically transformed cells from the non-genetically transformed cells using the method of the present invention.

The term "medium" includes any medium that is capable of sustaining the viability (such as growth) of the transformed cells while inhibiting or killing the non-transformed cells. For example, the medium may comprise typical ingredients of a growth medium but wherein those ingredients are in such an amount that the transformed cells are selectively grown. In accordance with the present invention, the medium may typically comprise at least a component, or a precursor therefor or a derivative thereof, according to the present invention.

The term "toxic" as used herein in relation to the non-transformed cells means that the component or derivative thereof or a precursor thereof has an adverse effect on the non-transformed cells or is metabolised to a derivative that has an adverse effect on the non-transformed cells. An example of an adverse effect is growth inhibition. The term also
5 includes death of the non-transformed cells.

The term "genetically transformed" includes transformation using recombinant DNA techniques.

10 The term "introducing the population of cells to a medium" means adding the population of cells to the medium or *vice versa*.

The component of the present invention may be derived from a precursor therefor.

15 Preferably, the precursor is present in a medium and the precursor is processed by the cells to the component - such as by use of naturally occurring enzymes and/or by use of heterologous enzymes (such as those expressed by recombinant DNA).

The term "vector" includes expression vectors and transformation vectors.

20

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred into a species - such as a plant by use of, for example, direct gene transfer techniques (such as
25 particle gun techniques for monocot transformation).

The term "tissue" includes tissue *per se* and organ.

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed when present in the organism.

5

Preferably the organism is a plant.

10

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

15

Preferably the transgenic organism is a plant.

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In a highly preferred embodiment, the transgenic organism (or part thereof) does not comprise the combination of a promoter and at least one of the first nucleotide sequence coding for the first enzyme according to the present invention, the second nucleotide sequence coding for the second enzyme according to the present invention and the third nucleotide sequence coding for the third enzyme according to the present invention, wherein both the promoter and the nucleotide sequence are native to that organism (or part thereof) and are in their natural environment. Thus, in this highly preferred embodiment, the present invention does not cover at least one of a native first nucleotide sequence coding for the first enzyme according to the present invention, a native second nucleotide sequence coding for the second enzyme according to the present invention and a native nucleotide sequence coding for the third enzyme according to the present invention in its natural environment when it is under the control of its native promoter which is also in its natural environment. In addition, in this highly preferred embodiment, the present invention does not cover the native enzyme according to the present invention (namely any one of the first enzyme according to the present invention, the second enzyme according to the present invention, the third enzyme according to the

present invention) when it is in its natural environment and when it has been expressed by its native nucleotide coding sequence which is also in its natural environment and when that nucleotide sequence is under the control of its native promoter which is also in its natural environment. In other words, it is preferred that the nucleotide sequence is
5 heterologous to the organism and/or is under the control of a heterologous promoter.

In accordance with a highly preferred aspect of the present invention, the component and/or the derivative thereof and/or the precursor thereof are not prepared *in situ* in the medium - i.e. they are actual ingredients that are added to make up the medium.

10

In one aspect of the present invention, the one or more components or derivatives thereof can act as a nutrient for the transformed cells.

However, in one aspect, the one or more components or derivatives thereof need not act
15 as the only possible nutrient for the transformed cells. Examples of other nutrients that may be used include any one or more of carbohydrates, sucrose, maltose, and other energy sources. In addition, or in the alternative, the transformed cells may be grown on other suitable growth media which do not contain the one or more components or derivatives. In this latter case, the transformed cells will not be selectable due to the
20 presence of just the sequences of the present invention - but they could also be selectable due to the presence of other selectable sequences.

Here, the term "nutrient" includes a substance that is capable of providing directly or indirectly (e.g. *via* a metabolite thereof) energy or atoms or molecules that are
25 beneficially useful for maintenance and/or growth and/or reproduction etc. of the cell, tissue, organ or organism.

For example, the term includes a substrate that can be beneficially metabolised and/or beneficially utilised in a metabolic pathway to enable the transformed cells to grow, to
30 proliferate or to be maintained in a viable form. Here, "beneficial" as used in relation to

the present invention means at least not causing an adverse, detrimental effect to the transformed cells.

5 In accordance with the present invention, a non-transformed cell is a cell that does not comprise at least one of the first nucleotide sequence according to the present invention, the second nucleotide sequence according to the present invention and the third nucleotide sequence according to the present invention.

10 The non-transformed cell of the present invention may even be a previously transformed cell that does not comprise at least any one of the first nucleotide sequence according to the present invention, the second nucleotide sequence coding according to the present invention and the third nucleotide sequence according to the present invention. The non-transformed cell of the present invention can, however, contain one or more heterologous nucleotide sequences, or one or more homologous nucleotide sequences
15 under the control of one or more heterologous transcriptional control elements.

The transformed cell of the present invention may contain one or more heterologous nucleotide sequences, or one or more homologous nucleotide sequences under the control of one or more heterologous transcriptional control elements.

20

In a highly preferred embodiment, the first nucleotide sequence is not in its natural environment. In this regard, the first nucleotide sequence may not be native (i.e. foreign) to the cell or organism. In addition, the first nucleotide sequence may be native to the cell or organism but wherein the first nucleotide sequence is operably linked to a
25 promoter that is heterologous to the first nucleotide sequence.

In accordance with the present invention there may be a plurality of first nucleotide sequences and/or second nucleotide sequences and/or third nucleotide sequences.

The or each first nucleotide sequence may be independently selected from DNA or RNA. Preferably, the or each first nucleotide sequence is DNA. More preferably, the or each first nucleotide sequence is recombinant DNA.

5 The second nucleotide sequence may be independently selected from DNA or RNA. Preferably, the or each second nucleotide sequence is DNA. More preferably, the or each second nucleotide sequence is recombinant DNA.

The third nucleotide sequence may be independently selected from DNA or RNA.
10 Preferably, the or each third nucleotide sequence is DNA. More preferably, the or each third nucleotide sequence is recombinant DNA.

The term "recombinant DNA" means DNA prepared by at least one step that utilises at least one recombinant DNA technique.

15

Thus, in a first broad aspect the present invention relates *inter alia* to the use of at least one nucleotide sequence encoding an expression product that affects galactose or a derivative thereof or a precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

20

In a second broad aspect the present invention relates *inter alia* to the use of the expression product of at least one nucleotide sequence that affects galactose or a derivative thereof or a precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

25

In a third broad aspect the present invention relates *inter alia* to galactose or a derivative thereof or a precursor thereof as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants).

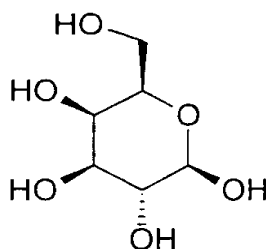
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Galactose is a hexose which has been demonstrated to be toxic to most plant species (eg. Farkas 1954; Hughes and Street 1974; Roberts et al. 1971).

Galactose has optical isomeric forms and can occur as a linear or a cyclic structure.

An example of a galactose molecule can be pictorially represented as:

5



Preferably the component is galactose, preferably D-galactose.

- 10 If the medium of the present invention comprises a precursor of galactose then preferably the precursor is capable of liberating free galactose or a derivative thereof.

Preferably the precursor is capable of liberating free galactose.

- 15 More preferably the precursor is capable of liberating free D-galactose.

If the medium of the present invention comprises a precursor of galactose then preferably one or more enzymes are present which can liberate free galactose or a derivative thereof from the precursor.

20

Preferably if the medium of the present invention comprises a precursor of galactose then preferably one or more enzymes are present which can liberate free D-galactose.

Preferably the enzyme(s) is at least a galactosidase.

25

Preferably the enzyme(s) is/are selected from one or more of α -galactosidases or β -galactosidases.

The enzyme can be present in the medium already or it can be prepared by the medium itself. Alternatively, the enzyme can be prepared by the transformed cell. Here, the enzyme can be native to the cell or it can be heterologous to the cell. The transformed
5 cell may also comprise an additional nucleotide sequence coding for an enzyme capable of releasing galactose from a precursor thereof.

Preferable examples of precursors of galactose include galactose containing compounds - such as lactoses, melibioses, raffinoses, stachyoses, verbascoses and galactinol.

10

More preferable precursors of galactose include α -lactose (β -D-galactopyranosyl [1 \rightarrow 4]- α -D-glucose, milk sugar), β -lactose, melibiose (6-O- α -D-galacto-pyranosyl-D-glucose), raffinose, stachyose, verbascose and galactinol and any other substrate which liberates free D-galactose upon hydrolysis by either α -galactosidases or β -galactosidases.

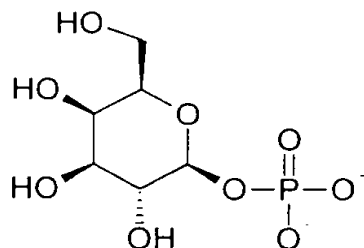
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Other examples of potentially useful precursors for use in the galactose selection method of the present invention are chemically derivatised forms of galactose, preferably chemical derivatives of D-galactose, from which free galactose can be liberated by use of appropriate techniques, such as enzyme action. By way of example, suitable chemical
20 derivatives are D-galactose pentaacetate and D-galactose methyl galactoside.

Alternatively or in addition, the medium may comprise a derivative of galactose.

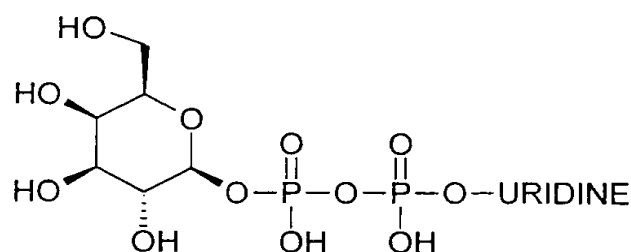
Preferable examples of such a derivative include galactose-1-phosphate and UDP-
25 galactose.

Galactose-1-phosphate can be pictorially represented as:



5

UDP-galactose can be pictorially represented as:



10

More preferable examples of such a derivative include D-galactose-1-phosphate and UDP-D-galactose.

15

Preferably the component is galactose-1-phosphate.

Preferably the precursor for the component is galactose.

20 Preferably each of the first expression product; the second expression product and the third expression product is independently selected from galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

Preferably each of the first expression product; the second expression product and the third expression product is independently selected from galactokinase (EC 2.7.1.6) obtainable from Petunia, yeast and other microorganisms, *Vicia faba*, *Phaseolus aureus*,
5 barley or corn, UTP-dependent pyrophosphorylase (EC 2.7.7.10) obtainable from Petunia, UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) obtainable from yeast and other microorganisms, UDP-galactose epimerase (EC 5.1.3.2) obtainable from Petunia, yeast and other microorganisms, fenugreek, wheat, sugarcane and various trees.

10 Preferably, each of the first expressable nucleotide sequence, the second expressable nucleotide sequence and the third expressable nucleotide is independently selected from expressable nucleotide sequences coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

15 Preferably, each of the first expressable nucleotide sequence, the second expressable nucleotide sequence and the third expressable nucleotide is independently selected from expressable nucleotide sequences coding for any one or more of galactokinase (EC 2.7.1.6) obtainable from Petunia, yeast and other microorganisms, *Vicia faba*, *Phaseolus*
20 *aureus*, barley or corn, UTP-dependent pyrophosphorylase (EC 2.7.7.10) obtainable from Petunia, UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) obtainable from yeast and other microorganisms, UDP-galactose epimerase (EC 5.1.3.2) obtainable from Petunia, yeast and other microorganisms, fenugreek, wheat, sugar cane and various trees.

25 In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence but not the optional second expressable nucleotide sequence and not the optional third expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence coding for UDP-galactose epimerase (EC 5.1.3.2) or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12). In this aspect, preferably the medium comprises UDP-galactose and/or galactose-1-phosphate, or derivatives thereof.

5

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence and the optional second expressable nucleotide sequence, but not the optional third expressable nucleotide sequence.

10 In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, and the second expressable nucleotide sequence is a nucleotide sequence that codes for UTP-dependent pyrophosphorylase (EC 2.7.7.10) and/or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12). In this aspect, preferably the medium comprises at
15 least galactose-1-phosphate. For example, the medium may comprise galactose-1-phosphate and UDP-galactose, or derivatives thereof.

In one preferred aspect of the present invention, the transformed cells comprise the first expressable nucleotide sequence and the optional third expressable nucleotide sequence,
20 but not the optional second expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, and the third expressable nucleotide sequence is a nucleotide sequence that codes for galactokinase
25 (EC 2.7.1.6). In this aspect, preferably the medium comprises at least galactose. For example, the medium may comprise galactose and UDP-galactose, or derivatives thereof.

In one preferred aspect of the present invention, the transformed cells comprise the first
30 expressable nucleotide sequence, the optional second expressable nucleotide sequence, and the optional third expressable nucleotide sequence.

In this aspect of the present invention, preferably the first expressable nucleotide sequence is a nucleotide sequence that codes for UDP-galactose epimerase, the second expressable nucleotide sequence is a nucleotide sequence that codes for UTP-dependent pyrophosphorylase (EC 2.7.7.10) and/or UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), and the third expressable nucleotide sequence is a nucleotide sequence that codes for galactokinase (EC 2.7.1.6). In this aspect, preferably the medium comprises at least galactose, or derivatives thereof. For example, the medium may comprise galactose and galactose-1-phosphate, or derivatives thereof. For example, the medium may comprise galactose and UDP-galactose, or derivatives thereof. For example, the medium may comprise galactose, galactose-1-phosphate and UDP-galactose, or derivatives thereof.

In accordance with the present invention, the medium can comprise one or more of the component, the derivative thereof and the precursor.

In order to prepare any one or more of the transformed cells, tissues, organs and organisms according to the present invention, the first expressable nucleotide sequence, the optional second expressable nucleotide sequence and the optional third expressable nucleotide sequence can be introduced into the original non-transformed cells by use of any one or more of a single construct, a single plasmid, or a single vector. Alternatively, the first expressable nucleotide sequence, the optional second expressable nucleotide sequence and the optional third expressable nucleotide sequence can be introduced into the original non-transformed cells by use of any one or more of: two or more constructs, two or more plasmids, or two or more vectors.

In accordance with the present invention any one or more of the first nucleotide sequence, the second nucleotide sequence and the third nucleotide sequence may comprise an intron.

In this respect, the presence of the intron within the coding portions of the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence may inactivate the expression products thereof *vis-a-vis* a prokaryote, such as a prokaryotic vector system used to generate the transformed cells, tissue, organs or organism.

The present invention therefore provides a method for selecting genetically transformed cells - such as cells into which a NOI has been incorporated - by providing the transformed cells with a selective advantage.

The method of the present invention is not dependent on the preparation of genetically transformed plants containing as a selection means a nucleotide sequence coding for antibiotic or herbicide resistance. Nevertheless, the method of the present invention can be used in conjunction with those earlier selection methods should the need arise - if for example it is desirable to prepare cells that have been or are to be transformed with a number of NOIs.

Also, the selection method of the present invention can be used in conjunction with one or more other known selection methods, such as those that are described in WO 93/05163 (the contents of which are incorporated herein by reference) and/or WO 94/20627 (the contents of which are incorporated herein by reference) and/or PCT/GB98/00367 (the contents of which are incorporated herein by reference), should the need arise - if for example it is desirable to prepare cells that have been transformed with a number of NOIs.

In addition, the selection method of the present invention can be used in conjunction with one or more other selection methods according to the present invention should the need arise - if for example it is desirable to prepare cells that have been transformed with a number of NOIs.

A further beneficial use of a combination of selection methods according to the present invention results in a very efficient multiple screening technique. In this regard, and by way of example, the medium in the first screen utilising the selection method of the present invention would contain added low amounts of the component or the derivative thereof. With this first screen, selectable transformed cells are selected over at least the majority of the non-transformed cells. Then should - for example - any non-transformed cells be accidentally be carried over in that first screen then a second screen can be carried out. In the second screen the selected population of cells are subjected to a second selection method according to the present invention but wherein the component or the derivative thereof is present in the medium in a high concentration. In the second screen, predominantly the transformed cells would remain viable.

With this combined aspect of the present invention, the population of cells of the earlier aspects of the present invention can therefore be a pre-selected (e.g. pre-screened) population of cells, wherein the population of cells has been prior selected by one or more selection methods, such as those according to the present invention.

This combined aspect of the present invention can be alternatively expressed as: a selection method for selecting from a population of cells one or more selectable genetically transformed cells, wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells; wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product; optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product; wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product; wherein the component can be present in an amount in a medium

that is toxic to the non-transformed cells; the method comprising (a) the step of introducing the population of cells to a medium; wherein the component is or will be in such an amount that is utilisable by the transformed cells but in an amount that is toxic to the non-transformed cells; wherein each of the first expression product and the optional
5 second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof; and (b) a subsequent step of introducing at least portion of the transformed cells to a medium that
10 comprises a higher concentration of the component or the derivative thereof or precursor thereof.

The present invention also encompasses compositions and kits useful for this combined aspect of the present - such as the nucleotide sequence or expression product thereof
15 according to the present invention, a first medium containing no component or derivative thereof, and a second medium comprising a high concentration of the component or derivative thereof.

Furthermore, the selection method of the present invention can be used in conjunction
20 (such as sequentially) with further selection methods wherein those further selection methods are a combination of one or more other selection methods according to the present invention and one or more known selection methods - such as those that are dependent on antibiotic or herbicide resistance and/or those that are disclosed in WO 93/05163 and/or WO 94/20627.

25

In the selection methods of WO 93/05163 and/or WO 94/20627, the *manA* gene from *Escherichia coli*, which encodes mannose-6-phosphate isomerase (E.C. 5.3.2.8.), was employed as a selectable marker. This selection marker is suitable for *inter alia* the transformation of *Solanum tuberosum*, conferring positive selection in the presence of
30 mannose. In more detail, the coding region of *manA* was ligated into a CaMV 35S expression cassette, and introduced into a binary vector for plant transformation

mediated by *Agrobacterium tumefaciens*. To allow comparison of kanamycin selection with selection on mannose, the vector also contained a gene for kanamycin resistance, *nptII*. In order to identify transformants, the construction also contained the *B*-glucuronidase histochemical marker, *uidA*. Stable integration of the *manA* gene was shown by Southern blotting. Extracts from plants transformed with this construct, and selected on mannose, were shown to have specific activities for mannose-6-phosphate isomerase some five hundred fold those of control plants. Expression of *manA* in transformed cells relieved the metabolic paralysis, usually caused by mannose, while also allowing it to serve as a source of carbohydrate for transformants. These effects combined to impose a stringent selection pressure in favour of transformed cells, which allowed the recovery of transformants with a very low frequency of escapes. The percentage of shoots which were shown to be transgenic after selection on mannose was approximately twice that of shoots selected on kanamycin. The transformants selected on mannose have proven to be stable over three generations of plants propagated from tubers.

Hence, the population of cells of the earlier aspects of the present invention can therefore be a pre-selected (e.g. pre-screened) population of cells, wherein the population of cells has been prior selected by one or more selection methods according to the present invention and/or one or more other selection methods.

In addition, or in the alternative, the transformed cells selected by the selection method of the present invention can be subsequently subjected to one or more selection methods according to the present invention and/or one or more other selection methods.

The present invention also provides an expression system that enables transformed cells to be selected by the selection method of the present invention. The expression system can be expressing or can be capable of expressing at least the first nucleotide sequence of the present invention. The expression system may be one or more of a vector, construct, plasmid, cell or organism.

If a cell is also to be transformed with a NOI then the expression system will comprise that NOI - which NOI may be present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. Alternatively the NOI may be present on or in a different vector, construct, plasmid, cell or organism as the first nucleotide sequence. Preferably, the NOI is present on or in the same vector, construct, plasmid, cell or organism as at least the first nucleotide sequence.

If a cell is to be transformed with one or more NOIs and one or more other genes for one or more other selection methods (such as another selection method according to the present invention and/or a known selection method) those other nucleotide sequences may be present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. Alternatively one or more of those other nucleotide sequences may be present on or in a different vector, construct, plasmid, cell or organism as at least the first nucleotide sequence. Preferably, those other nucleotide sequences are present on or in the same vector, construct, plasmid, cell or organism as the first nucleotide sequence. This allows for workers to easily prepare and easily select for cells that have been transformed with a number of NOIs etc.

In accordance with the present invention there may be a plurality of NOI(s).

The, or each, NOI may be independently selected from DNA or RNA. Preferably, the or each NOI is DNA. More preferably, the or each NOI is recombinant DNA.

As indicated above, the term "recombinant DNA" means DNA prepared by at least one step that utilises at least one recombinant DNA technique.

The term NOI means any desired nucleotide sequence for incorporation into the cells in question to produce genetically transformed cells. Introduction of nucleotide sequences into, for example, plants is widely practised, and it is believed that there are no limitations upon the nucleotide sequences whose presence may be selected (eg. detected) by use of the selection method of the present invention.

By use of the method of the present invention the presence of the NOI in the genetically transformed cells may be determined without the above-mentioned disadvantages associated with the selection systems relying solely on antibiotic resistance and/or herbicide resistance.

The NOI can be any nucleotide sequence of interest, such as any gene of interest. A NOI can be any nucleotide sequence that is either foreign or natural to the cell or organism (e.g. a particular plant) in question. Typical examples of a NOI include genes encoding proteins and enzymes that modify metabolic and catabolic processes. The NOI may code for an agent for introducing or increasing resistance to pathogens. The NOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues. The NOI may even code for a compound that is of benefit to animals or humans. Examples of NOIs include nucleotide sequences encoding any one or more of pectinases, pectin depolymerases, polygalacturonases, pectate lyases, pectin lyases, rhamno-galacturonases, hemicellulases, endo- β -glucanases, arabinases, or acetyl esterases, or combinations thereof, as well as antisense sequences thereof. The NOI may encode a protein giving nutritional value to a food or crop. Typical examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant proteins that have a more desirable amino acid composition (e.g. a higher lysine content than a non-transgenic plant).

The NOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and α -galactosidase. The NOI can be a gene encoding for any one of a pest toxin, an antisense transcript such as that for patatin or α -amylase, ADP-glucose pyrophosphorylase (e.g. see EP-A-0455316), a protease antisense, a glucanase or genomic β 1,4-endoglucanase.

The NOI may even code for or comprise an intron of a particular gene. Here the intron can be in sense or antisense orientation. In the latter instance, the particular gene could be DNA encoding β -1,4-endoglucanase. Antisense expression of genomic exon or intron

sequences as the NOI would mean that the natural β -1,4-endoglucanase expression would be reduced or eliminated but wherein the expression of a β -1,4-endoglucanase gene according to the present invention would not be affected.

5 The NOI may be the nucleotide sequence coding for the arabinofuranosidase enzyme which is the subject of PCT patent application PCT/EP96/01009. The NOI may be any of the nucleotide sequences coding for the ADP-glucose pyrophosphorylase enzymes which are the subject of PCT patent application PCT/EP94/01082. The NOI may be any of the nucleotide sequences coding for the α -glucan lyase enzyme which are described in
10 PCT patent application PCT/EP94/03397. The NOI may be any of the nucleotide sequences coding for the glucanase enzyme which are described in PCT patent application PCT/EP96/01008.

The NOI may also encode a permease or other transport factor which allows the
15 compound or precursor thereof or metabolised derivative thereof to cross the cell membrane and enter the transformed cells. Instead of facilitating uptake of a compound into a cell, the co-introduced nucleotide sequence may alternatively direct the component or precursor thereof or metabolised derivative thereof to a specific compartment - such as the plasma membrane or into the vacuole or the endoplasmic reticulum.

20

More than one NOI can be present.

The NOI can be co-introduced with the first nucleotide sequence according to the present invention.

25

The term "co-introduced" means that the two nucleotide sequences may be coupled to each other, or are otherwise introduced together, in such a manner that the presence of the co-introduced first nucleotide sequence in a cell indicates that the NOI has been introduced into the cell, i.e. if the first nucleotide sequence is shown to have been
30 introduced, the probability that the NOI has also been introduced is significantly

increased. The two nucleotide sequences may be part of the same genetic construct and may be introduced by the same vector.

The methods described herein may also be used when the co-introduced first nucleotide sequence and the NOI are introduced independently. This may be performed, for example, by using the same bacteria for incorporation of both genes and incorporating a relatively large number of copies of the NOI into the cells, whereby the probability is relatively high that cells which are shown to express the first nucleotide sequence will also contain and express the NOI.

In order for the introduced first nucleotide sequence and optional NOI to be expressed in the transformed cells, the genetic constructs containing the first nucleotide sequence and/or NOI will typically, but not necessarily, contain regulatory sequences enabling expression of the nucleotide sequences, e.g. known promoters and transcription terminators. Thus, the first nucleotide sequence will typically be associated with a promoter, which may be a constitutive or regulatable promoter, and the NOI will typically also be associated with a constitutive or regulatable promoter.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The promoter could additionally include one or more features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the *Shl*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements.

Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

5 The first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI may comprise one or more introns. In particular, if the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI encodes an expression product that can detrimentally affect a bacterium and all or a part (e.g. a plasmid thereof or therein) of that bacterium is
10 used either to propagate the NOI or as a means to transform the cells, then it may be desirable for that gene product to be inactive in the bacterium. One way of selectively inactivating the gene product in bacteria is to insert one or more introns into the nucleotide sequence of the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI, respectively. This intron
15 or those introns would not be removed after transcription in the bacterium but would be so removed in, for example, plants etc.

In a highly preferred embodiment, if the first nucleotide sequence and/or the second nucleotide sequence and/or the third nucleotide sequence and/or the NOI comprises at
20 least one intron, then that at least one intron is present in a highly conserved region of the first nucleotide sequence or the NOI.

Here, the term "intron" is used in its normal sense as meaning a nucleotide sequence lying within a coding sequence but being removable therefrom.

25 As mentioned above, the method of the present invention is particularly suitable for the selection of genetically transformed plant cells, thereby allowing identification and isolation of such cells without being essentially dependent on the use of selection genes coding for antibiotic or herbicide resistance.

The selection method of the present invention may be used for selecting cells *in vitro*. However, the selection method of the present invention may also be employed *in vivo* in the sense that it is possible to selectively grow transformed organisms - such as plants - from cells, tissues etc. that comprise the selection system of the present invention.

5 *In vivo* use of the selection method of the present invention is of particular importance in connection with genetic transformation performed on whole plants or on plant parts, in which the plants or plant parts comprise both transformed and non-transformed cells, since selection of the transformed cells can, in some instances, be achieved without
10 directly damaging the neighbouring non-transformed cells. For example, in some instances, the transformed cells have a selective advantage compared to the non-transformed cells - such as the ability to still form shoots - but the non-transformed cells suffer in the sense of being damaged or killed, as is the case with using antibiotics or herbicides.

15 In certain cases, such as when an improved selection frequency is desired, it may be advantageous for the cells to be transformed with a nucleotide sequence that is a selection gene different to the first nucleotide sequence. This additional, selection nucleotide sequence may be an additional gene coding for an enzyme (or other protein or
20 polypeptide) suitable for selection according to the present invention, or it may be a gene coding for an enzyme (or other protein or polypeptide) for a known selection method, eg coding for resistance to a antibiotic or herbicide or it may be a gene suitable for selection by the selection methods described in WO 93/05163 and/or WO 94/20627. Thus, genetically transformed cells may be selected using a combination of selection
25 techniques. For example, if the transformed cells also possessed genes coding for resistance to at least one antibiotic or herbicide, then the medium could additionally comprise at least one antibiotic or herbicide to which the transformed cells are resistant. In particular, we have found that the medium of the present invention does not impair the effectiveness of the known selection methods that rely on herbicide or antibiotic
30 resistance.

The selective advantage possessed by the transformed cells of the present invention may be any difference or advantage with regard to the non-transformed cells which allows the transformed cells to be readily identified and isolated from the non-transformed cells. This may, for example, be a difference or advantage allowing the transformed cells to be identified by simple visual means, i.e. without the use of a separate assay to determine the presence of a gene that provides the selection means.

As mentioned above, one aspect of the present invention relates to genetically transformed cells which have been selected according to the above method, in particular plant cells, as well as plants, progeny or seeds derived from or derivable from such genetically transformed plant cells. In particular, it is often an advantage that these cells are genetically transformed plant cells whose genome does not contain an introduced (i.e. non-native) nucleotide sequence coding for toxin-resistance, antibiotic-resistance or herbicide-resistance as a selection means. As explained above, there are concerns about whether it is safe to incorporate genes coding for eg antibiotic resistance in eg food plants. Genetically transformed plant cells selected by the method of the present invention which do not contain selection genes for eg antibiotic resistance, as well as plants, progeny and seeds derived from such cells, are therefore clearly advantageous in this respect.

The transformed cells may be prepared by techniques known in the art. For example, if the transformed cells are transformed plant cells reference may be made to EP-B-0470145 and CA-A-2006454.

Even though the selection method according to the present invention is not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare the transformed plant cells and transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

5 Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

10

Thus, in one aspect, the present invention relates to a vector system which carries a first nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

15

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

20

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture*
25 *Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The first nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the border sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the promoter or nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli.*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli.* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the first nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

Naturally, the present invention is not limited to just the use of *Agrobacterium* systems to transform plants. In this regard, other suitable techniques may be used - such as electroporation and/or particle bombardment (biolistics).

As reported in CA-A-2006454, a large number of cloning vectors are available which contain a replication system in *E. coli* and a selection means which allows a selection of the transformed cells. The vectors contain for example pBR322, the pUC series, the M13 mp series, pACYC 184 etc. In this way, the promoter or nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered and then analysed - such as by any one or more of the following techniques: sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted or selectively amplified by PCR techniques and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the first nucleotide sequence or construct according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblaserdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-

27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the first nucleotide sequence or the construct, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium.

When plant cells are constructed, these cells are grown and, optionally, maintained in a medium according to the present invention following well-known tissue culturing methods - such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc, but wherein the culture medium comprises a component according to the present invention.

Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting the transformed shoots and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.

Reference may even be made to Sprngstad *et al* (1995 Plant Cell Tissue Organ Culture 40 pp 1-15) as these authors present a general overview on transgenic plant construction.

In a highly preferred embodiment, the present invention is based on our finding that it is possible to use constructs comprising an expressable nucleotide sequence gene coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) to prepare transformed cells wherein the transformed cells can be selected from non-transformed cells.

In addition, the present invention also covers transgenic plants comprising the transformed cells or constructs of the present invention.

Thus, in a highly preferred embodiment the present invention covers transgenic plants comprising transformed cells or constructs that comprise an expressable nucleotide sequence gene coding for any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

In order to explain in more detail these highly preferred aspects of the present invention, reference shall be made to at least Figures 1 - 2, in which

Figure 1 is a schematic diagram of a metabolic pathway;

Figure 2 is a schematic diagram of a metabolic pathway.

Galactose metabolism in plants have been studied in *Petunia* which is one of the very few plant species being able to use galactose as carbon source to sustain growth (Dressler *et al.* 1982). The first step of galactose metabolism in *Petunia* as well as in yeast and other microorganisms is the phosphorylation of galactose to galactose-1-phosphate by galactokinase (EC 2.7.1.6) (see Figs. 1 and 2 below). The next step is the conversion to UDP-galactose which in *Petunia* can be performed by either a UTP-dependent pyrophosphorylase (EC 2.7.7.10) or a UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12) while in microorganisms only or predominantly the latter reaction occurs. The UDP-galactose is finally converted to UDP-glucose by UDP-galactose epimerase (EC 5.1.3.2). Thus, the conversion of galactose to a glucose-containing metabolite is irreversible.

The reason for the toxicity of galactose on plant cells is believed to be due to the absence of one or more of the enzymatic activities required for the conversion of galactose to UDP-glucose. Thus, in order to use galactose as selective agent, the transformed plant

cells must be supplemented with the lacking gene(s) so that enzymatic activities for the entire pathway from galactose to UDP-glucose are present.

However, it is likely that many plant species do not require the insertion of all 3 genes in the Leloir pathway as the absence of just one gene in this pathway will render the plant tissue sensitive to galactose.

The first enzyme in galactose metabolism is galactokinase which has been reported to be present in some plants such as *Vicia faba* (Dey 1983), *Phaseolus aureus* (Chan and Hassid 1975; Neufeld *et al.* 1960), barley and corn (Roberts *et al.* 1971). Roberts *et al.* (1971) studied the accumulation of galactose containing metabolites in corn roots fed with toxic levels of galactose and they found that free galactose and galactose-1-phosphate each accounted for close to 50% of the indentifiable metabolites. These data were in accordance with the hypothesis that the causal agent of galactose toxicity may be galactose-1-phosphate. As galactose is toxic to most plant species, this in turn implies that many plant species have galactokinase activity.

The next enzyme in the galactose metabolism is UTP-hexose-1-phosphate uridylyltransferase or UDP-glucose uridylyltransferase. Both of these enzymes - which are rare - may be found in *Petunia*.

In contrast to the two preceeding enzymes, the last enzyme, UDP-galactose epimerase, has been reported in a range of plant species such as fenugreek (Clermont and Percheron 1979), wheat (Fan and Feingold 1969), sugarcane (Maretzki and Thom 1978) and various trees (Dalessandro and Northcote 1977) in addition to the above mentioned species. The reason for the widespread occurrence of this enzyme is presumably related to the fact that its main function is the production of UDP-galactose from UDP-glucose for the synthesis of cell wall components.

Thus, in order to enable transformed plant tissue to metabolise galactose, it is likely that it is sufficient for some plant species to introduce just one gene eg. a UTP-hexose-1-phosphate transferase gene or a UDP-glucose uridylyltransferase gene which would then function as the selectable gene. However, for other plant species it may be required to
5 introduce two or even three genes involved in the metabolism of galactose. From a technical point of view, it presents no problems to use more than one gene as selectable gene.

Although a galactose selection system also employs a carbohydrate as selective agent, it
10 does not resemble 'positive selection' due to the high toxic effect of galactose.

The selection system described here is based on enabling transformed plant cells to metabolise D-galactose. This D-galactose can be supplied to the plant cells or tissues during selection as free D-galactose or as a D-galactose containing compound. Examples
15 of such D-galactose containing compounds are α -lactose (β -D-galactopyranosyl [1 \rightarrow 4]- α -D-glucose, milk sugar), β -lactose, melibiose (6-O- α -D-galacto-pyranosyl-D-glucose), raffinose, stachyose, verbascose and galactinol which all liberate free D-galactose upon hydrolysis by either α -galactosidases or β -galactosidases which are present in a wide range a plant species and tissues.

Other examples of potentially useful compounds for galactose selection are chemically derivatized D-galactose such as D-galactose pentaacetate and methyl galactoside, from which free D-galactose can be produced by the action of appropriate enzymes present in
20 plant cells.

It can also envisaged that the galactose selection system can be modified so that galactose-1-phosphate can be used as selective agent, in which case the selectable gene is most likely to be an UTP-hexose-1-phosphate uridylyltransferase gene or an UDP-glucose uridylyltransferase gene.
25

Yet another modification of the galactose selection system relates to the addition of other carbohydrates or other substances to the selection medium which affect the toxicity of galactose. Furthermore, it will also be obvious for those skilled in the art that changes in the employed galactose concentration in the course of the selection can affect selection efficiency.

Thus, the present invention provides a new selection method based on the novel use of galactose and/or a derivative thereof and/or a precursor thereof.

As galactose and the genes encoding the enzymes responsible for the metabolism of galactose are well-characterized, we believe that galactose would be suitable for use as a selective agent for the selection of transgenic cells, tissues, organs, organisms (such as plants), provided that the transgenic cells, tissues, organs, organisms (such as plants) acquire the appropriate genes for metabolising, detoxifying and/or tolerating galactose.

The present invention will now be described only by way of examples in which reference is made to the following Figures:

Figure 3 is presents sequence information; and

Figure 4 is a schematic diagram of a plasmid.

In more detail, Figure 3 is the complete nucleotide sequence of the *E. coli galT* gene with the deduced amino acid sequence shown below the nucleotide sequence. The primers used for the isolation of the gene by PCR are shown with arrows indicating the 5' to 3' direction.

In more detail, Figure 4 is a plasmid map of the plant transformation vector pVIC-GalT.

TRANSFORMATION STUDIES

The following examples demonstrate that the genes coding for galactokinase (EC 2.7.1.6) (*galK*), UTP-dependent pyrophosphorylase (EC 2.7.7.10) (*galP*), UDP-glucose-dependent uridyl transferase (EC 2.7.7.12) (*galT*), UDP-galactose epimerase (EC 5.1.3.2) (*galE*) gene can be used as a means to provide selection of transformed cells, such as transgenic potato or maize shoots, on or in media containing galactose or a derivative thereof as a selective agent. For convenience, the term *galX* has been used to denote any one or more of *galK*, *galP*, *galT*, *galE*.

EXAMPLE 1 - TRANSGENIC POTATO PLANTS

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents of each of which are incorporated herein by reference).

For the present studies, the following protocol was adopted.

Plasmid construction

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature 303 pp 179-180), was cultured on YMB agar ($K_2HPO_4 \cdot 3H_2O$ 660 mg l⁻¹, $MgSO_4$ 200 mg l⁻¹, NaCl 100 mg l⁻¹, mannitol 10 g l⁻¹, yeast extract 400 mg l⁻¹, 0.8% w/v agar, pH 7.0) containing 100 mg l⁻¹ rifampicin and 500 mg l⁻¹ streptomycin sulphate. Transformation with a plasmid containing *galX* under the control of a plant expressible promoter (such as the E35S promoter) was accomplished using the freeze-thaw method of Holters *et al* (1978 Mol Gen Genet 163 181-187) and transformants were selected on YMB agar containing 100 mg l⁻¹ rifampicin and 500 mg l⁻¹ streptomycin, and 50 mg l⁻¹ gentamycin sulphate. The resultant plasmid is called pVICTOR IV 35S *galX*. Transformation with a control construct lacking the *galX* gene was performed in the same manner. In addition, the T-DNA will include a

screenable marker gene encoding a screenable marker enzyme, such as the β -glucuronidase gene from *E. coli*, driven by a plant expressible promoter. This facilitates the optimization of the methods as transgenic cells/shoots/plants are more easily found using this marker (Jefferson *et al* 1987, EMBO J, 6:3901-3907).

5

Transformation of plants

Shoot cultures of *Solanum tuberosum* cv Saturna were maintained on LS agar containing Murashige Skoog basal salts (Sigma M6899) (Murashige and Skoog (1965) Physiol. Plant. 15: 473-497) with 2 μ M silver thiosulphate, and nutrients and vitamins as described by Linsmaier and Skoog (1965 Physiol. Plant. 18 100-127). Cultures were maintained at 25°C with a 16 h daily photoperiod. After approximately 40 days, subculturing was performed and the shoots cut into segments of approximately 8 mm length.

15

Shoot cultures of approximately 40 days maturity (5-6 cm height) were cut into 8 mm internodal segments and/or leaves were cut off and wounded by making 2-4 small cuts over the midrib of the leaf. These were then placed into liquid LS-medium containing *Agrobacterium tumefaciens* transformed with pVICTOR IV 35S *galX* ($A_{660} = 0.5$, pathlength 1 cm). Following incubation at room temperature for 30 minutes, the segments were dried by blotting on to sterile filter paper and transferred to LS agar (0.8% w/v containing 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid and 500 μ g l⁻¹ trans-zeatin. The explants were covered with filter paper, moistened with LS medium, and covered with a cloth for three days at 25°C. Following this treatment, the segments can be washed with liquid LS medium containing 800 mg l⁻¹ carbenicillin, and then transferred on to LS agar (0.8% w/v) containing 1 mg l⁻¹ trans-zeatin, 100 mg l⁻¹ gibberellic acid (GA3), with sucrose (eg 10-20 g l⁻¹). This agar contains galactose (eg such as in an amount of from about 0.5 - 5.0 g l⁻¹).

25

The segments were sub-cultured to fresh substrate each 3-4 weeks. In 3 to 4 weeks, shoots develop from the segments and the formation of new shoots continued for 3-4 months.

- 5 The regenerated shoots are maintained on substrate composed of LS-substrate, 0.002 mM silver thiosulphate and agar (8.0 g/l). Carbenicillin (800 mg/l) can be added if desired.

The transgenic plants may be verified by performing a β -glucuronidase assay on the leaf
10 tips of the surviving shoots according to Hodal *et al.* (Plant. Sci. (1992), 87: 115-122).

Alternatively, the transgenic genotype of the regenerated shoot may be verified by performing NPTII assays (Radke, S. E. *et al.*, Theor. Appl. Genet. (1988), 75: 685-694) or by performing PCR analysis according to Wang *et al.* (1993, NAR 21: 4153-4154).

15

The shoots (height approximately 2-3 cms) were transplanted from rooting substrate to soil and placed in a growth chamber (21°C, 16 hour light 200-400 μ E/m²/sec).

When the plants were well established they were transferred to the greenhouse, where
20 they were grown until tubers had developed and the upper part of the plants were senescing.

Harvesting

- 25 The potatoes were harvested after about 3-6 months and then analysed.

The transformed shoots can be distinguished from the non-transformed shoots by adding galactose to their substrate of leaf tips cultured in in-vitro. After harvest of the shoots, the transformed shoots can be selected by adding amounts of galactose to the shoot
30 medium. The transformed shoots will be resistant to galactose and will survive as opposed the non-transformed shoots which will be inhibited in growth.

Analysis of Transformants

In order to confirm the integration of *galX*, genomic DNA may be isolated by the method of Dellaporta *et al* (1983 Plant Mol Biol Rep 1 19-21) and samples of this DNA, digested with *EcoRI*, subjected to electrophoresis in an 0.8% w/v agarose gel and transferred to Hybond N+ membranes (Amersham) by Southern blotting (Southern, 1975 J Mol Biol 98 503-517). Probes for the coding region of *galX* may be used as templates for random primed synthesis of ³²P-labelled probe after the method of Feinberg and Vogelstein (1983 Anal Bioch 137 266-267) and hybridised to the Southern blots at high stringency (65°C, 0.1 x SSC).

Selection of transgenic shoots was accomplished using a selection medium according to the present invention.

Transgenic shoots are obtained on selection media according to the present invention indicating that the selection medium can be varied significantly and remain useful for the selection of transgenic shoots.

EXAMPLE 2 - TRANSGENIC MAIZE PLANTS

Introduction

Since the first publication of production of transgenic plants in 1983 (Leemans, 1993 Biotechnology 11 s22), there have been numerous publications of production of transgenic plants including especially dicotyledon crop plants.

Until very recently there were very few reports on successful production of transgenic monocotyledonary crop plants. This relatively slow development within monocots were due to two causes. Firstly, until the early 1980s, efficient regeneration of plants from cultured cells and tissues of monocots had proven very difficult. This problem was

ultimately solved by the culture of explants from immature and embryogenic tissue, which retain their morphogenic potential on nutrient media containing plant growth regulators. Secondly, the monocots are not a natural host for *Agrobacterium tumefaciens*, meaning that the successful developed techniques within the dicots using their natural vector *Agrobacterium tumefaciens* was unsuccessful for many years in the monocots.

Nevertheless, it is now possible to successfully transformation and produce fertile transgenic plants of maize using methods such as: (1) Silicon Carbide Whiskers; (2) Particle Bombardment; (3) DNA Uptake by PEG treated protoplast; or (4) DNA Uptake in Electroporation of Tissue. Each of these methods - which are reviewed by Thompson (1995 Euphytica 85 pp 75-80) - may be used to prepare *inter alia* transgenic maize according to the present invention.

In particular, the particle Gun method has been successfully used for the transformation of monocots. However, EP-A-0604662 reports on a different method of transforming monocotyledons. The method comprises transforming cultured tissues of a monocotyledon under or after dedifferentiation with *Agrobacterium* containing a super binary vector as a selection means a hygromycin-resistant gene was used. Production of transgenic calli and plant was demonstrated using the hygromycin selection. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

Subsequent to the method of EP-A-0604662, EP-A-0672752 reports on non-dedifferentiated immature embryos. In this regard, both hygromycin-resistance and PPT-resistance genes were used as the selection means, with PPT giving rise to 10% or more independent transformed plants. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

To date, it would appear that transgenic maize plants can be successfully produced from easily-culturable varieties - such as the inbred line A188. In this regard, see the

teachings of Ishida *et al* (1996 Nature Biotechnology 14 pp 745-750). The method disclosed by these workers may be used to prepare *inter alia* transgenic maize according to the present invention.

- 5 Vasil (1996 Nature Biotechnology 14 pp 702-703) presents a further review article on transformation of maize.

Even though it is possible to prepare transformed maize by use of, for example, particle Gun mediated transformation, for the present studies the following protocol is adopted.

10

Plasmid construction

The same protocol as outlined above is adopted. In this respect, the protocol also uses pVICTOR IV 35S *galX*. Likewise, transformation with a control construct lacking the
15 *galX* gene was performed in the same manner.

Isolation and cocultivation of explants

Immature embryos of, for example, maize line A188 of the size between 1.5 to 2.5 mm
20 were isolated and cocultivated with *Agrobacterium tumefaciens* strain LBA 4404 in N6-AS for 2-3 days at 25°C under illumination. Thereafter, the embryos were washed with sterilized water containing 250 mg/l of cefotaxime and transferred to an LS medium and 250 mg/l cefotaxime and galactose in concentrations of up to 100 mg/l (the medium is hereafter called LSS2).

25

Conditions for the selection of transgenic plants

The explants were cultured for three weeks on LSS2 medium and then transferred to an LS medium containing galactose and optionally cefotaxime. After three weeks on this
30 medium, green shoots were isolated and tested for β -glucuronidase (Jefferson *et al* 1987, EMBO J 6:3901-3907) activity.

Rooting of β -glucuronidase positive shoots

β -glucuronidase positive shoots were transferred to an MS medium containing 2 mg/l for
5 rooting. After four weeks on this medium, plantlets are transferred to pots with sterile
soil for acclimatisation.

Selection of transgenic shoots was accomplished using a selection medium according to
the present invention.

10

After four weeks the shoots were harvested and all explants were transferred to fresh
selection medium (same composition) and after another four weeks of selection the last
shoots were harvested.

15 After harvest, the shoots were analysed for GUS activity using the histochemical assay.

Transgenic shoots are obtained on selection media according to the present invention
indicating that the selection medium can be varied significantly and remain useful for the
selection of transgenic shoots.

20

EXAMPLE 3 - TOXICITY OF GALACTOSE TO VARIOUS PLANT SPECIES

In order to be useful for selection, the selective component (otherwise called selective
agent) should not be able to sustain significant growth and preferably have adverse/toxic
25 effects to the non-transformed cells/tissues. To assess the applicability of galactose
selection following transformation, a number of plant species representing a range of
different botanical families were tested for sensitivity to galactose, using explants which
may be suitable for transformation experiments.

Galactose was unable to sustain growth of any of the tested plant species (wheat, sunflower, oil seed rape, potato, sugar beet, pea and Petunia) with varying levels of toxicity. The only fairly tolerant species was Petunia, in accordance with expectations as Petunia has been reported to have all necessary enzymatic activities for metabolising galactose (Dressler *et al.* 1982. Z. Pflanzenphysiol. 107: 409-418).

3.1. Wheat (*Triticum aestivum*)

As an example of a monocotyledonous species, wheat was studied. Immature wheat embryos (2-3 weeks old, a total of 161) were carefully removed from the seed and were cultivated on Gamborg B5 medium (Gamborg *et al.* 1968. Exp. Cell Res. 50: 151-158) supplemented with 9.0 g/l Bacto agar, 20 g/l sucrose and galactose according to the experiment. The embryos were cultivated in the dark for about 10 days where germinated embryos were transferred to fresh medium of the same composition and cultivated in a 16 h/8 h day/night regime for 4-5 weeks. The effect of galactose was evaluated by the number of germinated embryos and by measuring the length of the leaf on these embryos, relative to the control medium (no galactose).

Galactose concentration (g/l)	0	2.5	5.0	7.5	10	15
Germination of embryos	100	43	29	0	14	0
Average shoot length	100	89	67	0	33	0

The data in the above table clearly demonstrate that galactose has a strong inhibiting effect on the immature embryos as both germination and growth of the shoots were significantly reduced. The galactose selection method of the present invention can be used for the production of transgenic wheat.

Roberts *et al.* (1981. Plant Physiol. 48: 36-42) found that galactose was toxic to tissues of maize (*Zea maize*) and of barley (*Hordeum vulgare*). This indicates that the galactose selection method of the present invention may also be useful for the selection of transgenic shoots of these species.

3.2. Sunflower (*Helianthus annuus*)

The preparation of apical explants useful for the transformation of sunflower were modified according to Knittel *et al.* (1994. Plant Cell Rep. 14: 81-86). The seed coats of 2 d old seedlings were gently removed and the cotyledons, the emerging root and the top of the apex were removed. The remaining part of the apex was cut off and divided into two halves and cultivated according to Knittel *et al.*, with 20 g/l sucrose in the medium and galactose according to the experiment. After 4-5 weeks of culture at a 16 h/8 h day/night regime, growth was monitored by determining the fresh weight of the explants (above controls grown on a medium devoid of any carbohydrate source).

Galactose concentration (g/l)	0	5.0	7.5	10	15
Fresh weight (mg/explant)	144	28	0	0	0

The data show that galactose is toxic to sunflower explants. The data indicate that the galactose selection method of the present invention may be employed successfully for the selection of transgenic sunflower shoots.

3.3. Oil seed rape (*Brassica napus*)

Hypocotyl explants useful for the transformation of oil seed rape were prepared using a protocol modified after Radke *et al.* (1988. Theor. Appl. Genet. 78: 161-168). Hypocotyls of 5 d old seedlings were cut into 1 cm segments and transferred to modified

MS-medium (Murashige and Skoog 1962. *Physiol. Plant.* 15: 473-497) containing sucrose and galactose according to the experiment. After 3-4 weeks, shoots were harvested and the explants were transferred to fresh medium of the same composition. This was repeated for 3 more harvests and the total number of regenerated shoots formed on each medium was calculated (see table below).

Sucrose concentration (g/l)	0	5.0	10	20
0 g/l galactose	0	-	-	60
2.5 g/l galactose	0	1	7	26
5.0 g/l galactose	0	0	3	22

The data in the above Table clearly show that galactose is toxic to oil seed rape. Hence, the data indicate that the galactose selection method of the present invention may be used for the selection of transgenic shoots of oil seed rape and presumably related species. It is expected that higher concentrations of galactose are even more toxic.

The data also demonstrate that the toxic effect of galactose strongly interacts with sucrose as increasing concentrations of sucrose progressively reduces the toxic effect of galactose. Hence, for some applications, preferably galactose is present in low amounts of sucrose.

3.4. Potato (*Solanum tuberosum*)

Young leaves useful for the transformation of potato were prepared from leaves of 28 d old in vitro shoot cultures. The leaves were cut off avoiding the meristem at the leaf corner and were cut twice across the midvein. The explants were then put on modified MS-medium (Murashige and Skoog 1962. *Physiol. Plant.* 15: 473-497) containing various concentrations of sucrose and galactose. After 3 weeks, shoots were harvested and the explants were transferred to fresh medium of the same composition. This was repeated for 2 more harvests and the total number of explants with regenerated shoots formed on each medium was calculated (see table below).

Sucrose concentration (g/l)	0	5.0	10	20	30
0 g/l galactose	0	-	-	-	14
2.5 g/l galactose	1	0	0	7	-
5.0 g/l galactose	0	0	0	0	-

The data in the above Table clearly show that galactose is toxic to potato. The data indicate that the galactose selection method of the present invention may be used for the selection of transgenic shoots of potato and presumably related species. Like oil seed rape, these data also demonstrate that the toxic effect of galactose strongly interacts with sucrose as increasing concentrations of sucrose progressively reduces the toxic effect of galactose. We believe that such interaction is likely to take place also for many other plant species. We also believe that such interaction will also be observed with other carbohydrates than sucrose, such as glucose, fructose and maltose etc. Hence, for some applications, preferably galactose is present in low amounts of carbohydrate (i.e. in amounts that do not affect substantially the effect of the galactose).

3.5. Sugar beet (*Beta vulgaris*)

Cotyledonary explants useful for the transformation of sugar beet were prepared by excising the cotyledons from 3-4 weeks old seedlings (Joersbo *et al.* 1998. Mol. Breeding 4: 111-117). The leaves were transferred to modified MS-medium (Murashige and Skoog 1962. Physiol. Plant. 15: 473-497) containing 20 g/l sucrose and various concentrations galactose.

After 4-5 weeks, the number of shoots and the fresh weight of the explants were determined and a significant reduction of both growth parameters was observed. The data indicate that the galactose selection method of the present invention may be used for the selection of transgenic shoots of sugar beet and presumably related species.

3.6. Pea (*Pisum sativum*)

Hypocotyl explants useful for the transformation of pea were prepared by excising the hypocotyl from 7 d old seedlings and transferred to a medium, according to Nielsen *et al.* (1991. Physiol. Plant 82: 99-102), containing various concentrations of sucrose and galactose. After 5 weeks, the growth of the explants were evaluated. The results showed that galactose was unable to sustain any growth of the explants, as opposed to the control medium containing 20 g/l sucrose (no galactose) where the explants produced a many-fold increased fresh weight. The data indicate that the galactose selection method of the present invention may be used for the selection of transgenic shoots of pea and presumably related species.

EXAMPLE 4 GALACTOSE SELECTION EMPLOYED FOR THE SELECTION OF TRANSGENIC SHOOTS

Introduction

5

In one preferred embodiment, it is desirable to use up to 3 genes encoding enzymes useful for the conversion of galactose to UDP-glucose. However, in some instances we have found that only one gene may be used. An example of such a gene is that coding for a UTP-dependent pyrophosphorylase (EC 2.7.7.10) or a UDP-glucose-dependent
10 uridyl transferase (EC 2.7.7.12). In some instances, these individual genes may be sufficient for the selection of transgenic shoots of many plant species because both galactokinase (EC 2.7.1.6) and UDP-galactose epimerase (EC 5.1.3.2) occur naturally in many plant species.

15 In this example, a further example (i.e. in addition to Example 1) the successful selection of transgenic potato shoots is described. These particular experiments used a transformation vector including an *E. coli* gene (*galT*) encoding UDP-glucose-dependent uridyl transferase (EC 2.7.7.12).

20 The fact that it was possible select transgenic potato shoots on galactose containing media is in accordance with the toxicological test where potato was found to be sensitive to galactose. Consequently, it is expected that transgenic shoots of other plant species that are sensitive to galactose also can be selected using galactose selection.

25 4.1. Transformation vector

An *E. coli galT* gene encoding galactose-1-phosphate uridyltransferase has been cloned, sequenced and introduced into a plant transformation vector. The cloned gene consists of 1060 base pairs and codes for a 348 amino acid protein. Transformation of a *galT*
30 deficient *E. coli* strain resulted in colonies able to grow on galactose as sole carbon source, indicating that the cloned gene expresses a functional enzyme capable of

complementing the *galT* deficiency. The gene was furnished with an enhanced 35S promoter and a 35S terminator and inserted into a plant transformation vector based on our pVIC vector, into which a GUS gene was also inserted.

5 Cloning and characterization of the *E. coli galT* gene.

The *galT* gene of *E. coli* was isolated and cloned by PCR from genomic DNA isolated from an *E. coli* strain that can metabolise galactose. The *galT* gene was found in the GenBank Sequence Database, and the nucleotide sequence was used to design PCR
10 primers for the amplification of the coding region of the gene. To facilitate cloning into our expression vectors the upstream primer was designed such that a *PstI* site was added to the 5' end of the primer, and the downstream primer was similarly designed with a *SalI* site. A 1050 base pair DNA fragment obtained after PCR was cloned into the TA cloning vector pCR 2.1-TOPO. Five of the clones were completely sequenced and one
15 clone was selected for further use. The nucleotide sequence of the selected clone (Fig. 3) was identical to that of Cornwell *et al.* (1987. Nucl. Acids Res. 15: 8116). Translation of the cloned *galT* gene revealed an open reading frame of 1047 base pairs encoding a 348 amino acid protein (Fig. 3) in agreement with the published sequence (Cornwell *et al.* 1987. Nucl. Acids Res. 15: 8116).

20

Construction of a plant transformation vector with the *galT* gene

A plant transformation vector called pVIC for *Agrobacterium* mediated transformation was used. pVIC contains the following features: Right- and left border sequences (RB
25 and LB) are from the *A. tumefaciens* plasmid pTiT37 (Yadav *et al.* 1982. Proc. Natl. Acad. Sci. 79: 6322-6326). The replication origin (pUC Ori) for replication and maintenance in *E. coli* is from the plasmid pUC19 (Yanish-Perron *et al.* 1985. Gene 33: 103-119). The replication origin (pVS1 Ori) for replication and maintenance in *A. tumefaciens* is from the *Pseudomonas* plasmid pVS1 (Itoh *et al.* 1984. Plasmid 11:
30 206-220). The bacterial spectinomycin/streptomycin resistance gene (Spec/Strep) is from the transposon Tn7 (Fling *et al.* 1985. Nucl. Acids Res. 19: 7095-7106) and is

fused to the hybrid trp-lac promoter (Amman *et al.* 1983. Gene 25: 167-178) for efficient bacterial expression. A β -glucuronidase gene (Jefferson *et al.* 1986. Proc. Natl. Acad. Sci. 83: 8447-8451) containing an intron of the ST-LS1 gene from potato, preventing expression in bacteria (Vancanneyt *et al.* 1990. Mol. Gen. Genet. 220: 245-250) was furnished with a 35S promoter and a 35S terminator and was introduced into pVIC. This construct was used as a vector for the *galT* gene. The cloned *galT* gene was cloned between an enhanced 35S promoter (E35S PR) and the 35S terminator (35S t) (Key *et al.* 1987. Science 236: 1299-1302 and Odell *et al.* 1985. Nature 313: 810-812) and this expression cassette was ligated into above mentioned vector. This resulted in the final plant transformation vector pVIC-galT, as shown schematically in Fig. 4.

4.2. Transformation of potato explants

Young leaves useful for the transformation of potato were prepared from leaves of 28 d old in vitro shoot cultures. The leaves were cut off avoiding the meristem at the leaf corner and were cut twice across the midvein. The explants were inoculated for 30 min. with a suspension of *Agrobacterium tumefaciens* strain EHA101 ($OD_{660}=0.5$) harbouring the transformation vector described above. After inoculation, the explants were dried carefully on filterpaper and left for co-cultivation for 3 d in dim light.

4.3. Selection of transgenic potato shoots on galactose containing media

After co-culture, the explants were transferred to selection medium consisting of MS-medium (Murashige and Skoog 1962. Physiol. Plant. 15: 473-497) supplemented with 500 mg/l carbenicillin and various concentrations of sucrose and galactose. After 3 and 6 weeks, shoots were harvested and transgenicity was evaluated by subjecting the harvested shoots to the assay for β -glucuronidase activity (Jefferson *et al.* 1987. Plant Mol. Biol. Rep. 5: 387-405), a marker enzyme encoded by a gene placed adjacent the selective gene on the T-DNA of the transformation vector (Fig. 4). The number of transgenic shoots isolated on the various selection media is indicated in the table below.

Galactose concentration (g/l)	Sucrose concentration (g/l)	No. of transgenic shoots	Transformation frequency (%)
0	20	0	0
1.25	10	3	6.0
1.25	15	7	14.0
1.25	20	3	6.0
2.5	10	1	2.0
2.5	15	0	0
2.5	20	4	8.0

The data show that it is possible to select transgenic shoots of a plant, transformed with a gene encoding an enzyme that enables or enhances the ability of the transformed shoots to reduce the toxicity of galactose.

In an other experiment, a few transgenic potato shoots (4%) were harvested on a medium devoid of galactose. Without wishing to be bound by theory we believe that this selective effect may be related to a stimulating effect of the inserted selective gene *per se* and/or the expression product thereof *per se* on the regeneration potential of the transgenic cells.

In combination with the toxicological studies, the fact that transgenic potato shoots can be selected on galactose-containing media suggests that transgenic shoots of other plant species that are unable to grow on or inhibited by galactose also may be selected.

- 5 Without wishing to be bound by theory, we also believe that the degree of selectivity may differ due to the choice of the selection media - which may contain galactose and/or metabolic precursors and/or metabolic derivatives and/or derivatives of galactose, alone or in combination with other carbohydrate/energy source(s) such as sucrose, glucose, fructose, maltose which may or may not affect the effect of galactose. By way of
10 example, it may be possible to yield similar or even substantially higher transformation frequencies.

Without wishing to be bound by theory, we also believe that the degree of selectivity may differ among different varieties/cultivars/subspecies of a plant species. Also, other
15 physical and/or chemical and/or biological parameters affecting the effect of galactose may be expected to have an impact on the number of selectable transgenic shoots, possibly increasing the transformation frequencies significantly.

EXAMPLE 5 - TRANSGENIC RAPE SEED

20

Hypocotyl explants of oil seed rape were prepared using a protocol modified according to Radke *et al.* (1988. Theor. Appl. Genet. 78: 161-168). Hypocotyls of 5 d old seedlings were cut into 1 cm segments and inoculated with a suspension of *Agrobacterium tumefaciens* strain EHA101 ($OD_{660}=0.1-0.5$, prepared according to
25 standard procedures). After inoculation, the explants were dried carefully on filterpaper before co-cultivation.

Selection of transgenic oil seed rape on galactose containing media

After co-culture, the explants were transferred to selection medium consisting of modified MS-medium (Murashige and Skoog 1962. *Physiol. Plant.* 15: 473-497) supplemented with 500 mg/l carbenicillin and various concentrations of sucrose and galactose. After 7-8 weeks, shoots were harvested and transgenicity was evaluated by subjecting the harvested shoots to the assay for β -glucuronidase activity (Jefferson *et al.* 1987. *Plant Mol. Biol. Rep.* 5: 387-405), a marker enzyme encoded by a gene placed adjacent the selective gene on the T-DNA of the transformation vector (Fig. 3). The number of harvested shoots which were more than 50% β -glucuronidase-positive is indicated in the table below.

Galactose concentration (g/l)	Sucrose concentration (g/l)	No. of transgenic shoots	Transformation frequency (%)
0	20	0	0
2.5	10	4	1.0

The data show that it is possible to select transgenic shoots of oil seed rape, transformed with a gene encoding an enzyme that enables or enhances the ability of the transformed shoots to reduce the toxicity of galactose.

Without wishing to be bound by theory, we also believe that the degree of selectivity may differ due to the choice of the selection media - which may contain galactose and/or metabolic precursors and/or metabolic derivatives and/or derivatives of galactose, alone or in combination with other carbohydrate/energy source(s) such as sucrose, glucose, fructose, maltose which may or may not affect the effect of galactose. By way of example, it may be possible to yield similar or even substantially higher transformation frequencies.

Without wishing to be bound by theory, we also believe that the degree of selectivity may differ among different varieties/cultivars/subspecies of a plant species. Also, other physical and/or chemical and/or biological parameters affecting the effect of galactose may be expected to have an impact on the number of selectable transgenic shoots, possibly increasing the transformation frequencies significantly.

SUMMATION

In summation, therefore, the present invention relates to a selection method for selecting from a population of cells one or more selectable genetically transformed cells. The population of cells comprises selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressible nucleotide sequence encoding a first expression product. Each of the selectable genetically transformed cells optionally comprises an optional second expressible nucleotide sequence encoding a second expression product and/or an optional third expressible nucleotide sequence encoding a third expression product. In the method a component is utilisable by the selectable genetically transformed cells by action of the first expressible nucleotide sequence or the first expression product and optionally by action of the optional second expressible nucleotide sequence or the optional second expression product and/or by action of the optional third expressible nucleotide sequence or the optional third expression product. The component can be present in an amount that is toxic to the non-transformed cells. The selection method comprises the step of introducing the population of cells to a medium, wherein the medium comprises the component and/or a derivative thereof and/or a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells. In the method, each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof. In the method, the component and/or the derivative

thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

The present invention also relates to a number of other aspects which also contain some
5 or all of the same distinguishing technical features - such as: a composition comprising a
population of cells comprising selectable genetically transformed cells and possible non-
transformed cells, and a medium; a population of cells comprising selectable genetically
transformed cells and possible non-transformed cells; a selectable genetically
transformed cell; an organism comprising a selectable genetically transformed cell; a
10 construct for genetically transforming a non-transformed cell to produce a selectable
genetically transformed cell.

In one aspect of the present invention, the component is present in the medium.

15 Hence, this aspect of the present invention can be expressed as:

a selection method for selecting from a population of cells one or more selectable
genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells
and possible non-transformed cells;

20 wherein each of the selectable genetically transformed cells comprises a
first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises
an optional second expressable nucleotide sequence encoding a second expression
product and/or an optional third expressable nucleotide sequence encoding a third
25 expression product;

wherein a component is utilisable by the selectable genetically transformed cells
by action of the first expressable nucleotide sequence or the first expression product and
optionally by action of the optional second expressable nucleotide sequence or the
optional second expression product and/or by action of the optional third expressable
30 nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and optionally a derivative thereof
5 and/or a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an
10 enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

15 Likewise, this aspect of the present invention also relates to a number of other aspects which also contain some or all of the same distinguishing technical features - such as: a composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells, and a medium; a population of cells comprising selectable genetically transformed cells and possible non-transformed
20 cells; a selectable genetically transformed cell; an organism comprising a selectable genetically transformed cell; a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell.

For some applications the component is detoxifiable by the selectable genetically
25 transformed cells.

One aspect of this aspect of the present invention can be expressed as:

a selection method for selecting from a population of cells one or more selectable genetically transformed cells,

30 wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component or a derivative thereof or a precursor thereof when present in a detoxifiable amount in a medium is detoxifiable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component or the derivative thereof or the precursor thereof can be present in an amount in a medium that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and/or the derivative thereof and/or the precursor therefor and in an amount that is detoxifiable by the transformed cells but in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

25

Likewise, this aspect of the present invention also relates to a number of other aspects which also contain some or all of the same distinguishing technical features - such as: a composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells, and a medium; a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; a selectable genetically transformed cell; an organism comprising a selectable

30

genetically transformed cell; a construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell.

5 In one preferred aspect, the component is prepared *in situ* in the cells by processing a precursor therefor or a derivative thereof that was present in a medium in which the cells are or were present.

Hence, this preferred aspect can be expressed as:

10 a selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

15 optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

20 wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

25 wherein the component can be present in an amount that is toxic to the non-transformed cells;

the method comprising the step of introducing the population of cells to a medium,

30 wherein the medium comprises a derivative of the component and/or a precursor of the component and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

5 wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

Likewise, this preferred aspect of the present invention also relates to a number of other aspects which also contain some or all of the same distinguishing technical features -
10 such as: a composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells, and a medium; a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; a selectable genetically transformed cell; an organism comprising a selectable genetically transformed cell; a construct for genetically transforming a non-transformed
15 cell to produce a selectable genetically transformed cell.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope
20 and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of
25 the following claims.

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CLAIMS

1. A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

5 wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises
10 an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and
15 optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;
20 the method comprising the step of introducing the population of cells to a medium,

wherein the medium comprises the component and/or a derivative thereof and/or a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

25 wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the derivative thereof and/or the precursor thereof
30 is galactose or a derivative thereof or a precursor thereof.

2. A composition comprising a population of cells comprising selectable genetically transformed cells and possible non-transformed cells; and a medium;

wherein each of the selectable genetically transformed cells comprises a first expressible nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressible nucleotide sequence encoding a second expression product and/or an optional third expressible nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressible nucleotide sequence or the first expression product and optionally by action of the optional second expressible nucleotide sequence or the optional second expression product and/or by action of the optional third expressible nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein the medium comprises the component and/or a derivative thereof and/or a precursor thereof and in an amount such that the component is or will be in an amount that is utilisable by the transformed cells but wherein the component is or will be in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

3. A population of cells comprising selectable genetically transformed cells and possible non-transformed cells;

wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

5 optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

10 wherein a component is utilisable by the selectable genetically transformed cells by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

15 wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof; and

20 wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

4. A selectable genetically transformed cell comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable
25 nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells by action of the the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the
30 optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
5 and

wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

10 5. An organism comprising a selectable genetically transformed cell according to claim 4.

6. A construct for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell;

15 the construct comprising a first expressable nucleotide sequence encoding a first expression product; and optionally a second expressable nucleotide sequence encoding a second expression product and/or a third expressable nucleotide sequence encoding a third expression product;

wherein a component is utilisable by the selectable genetically transformed cells
20 by action of the first expressable nucleotide sequence or the first expression product and optionally by action of the optional second expressable nucleotide sequence or the optional second expression product and/or by action of the optional third expressable nucleotide sequence or the optional third expression product;

wherein the component can be present in an amount that is toxic to the non-transformed cells;
25

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof;
and

30 wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

7. A vector comprising the construct according to claim 6.

8. A plasmid comprising the construct according to claim 6.

5

9. A kit comprising a construct according to claim 6 or a vector according to claim 7 or a plasmid according to claim 8 for genetically transforming a non-transformed cell to produce a selectable genetically transformed cell; and a medium.

10 10. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vitro* within a culture.

11. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are *in vivo* within an organism.

15

12. The invention according to any one of the preceding claims wherein the selectable genetically transformed cell/cells is/are selectable genetically transformed plant cell/cells.

20 13. The invention according to any one of the preceding claims wherein an additional nucleotide sequence is present and wherein the additional nucleotide sequence codes for a nucleotide sequence of interest.

25 14. The invention according to any one of the preceding claims wherein the component is present in the medium.

15. The invention according to any one of claims 1 to 13 wherein the component is prepared *in situ* in the cell from a precursor that was present in the medium.

16. A plant prepared from or comprising the invention according to any one of the preceding claims, preferably wherein the plant is capable of providing a feed, foodstuff to humans or animals.

5 17. A plant according to claim 14 wherein the plant is any one of rape seed, potato or maize.

18. A plant comprising one or more heterologous enzymes, wherein the heterologous enzymes are any one or more of galactokinase (EC 2.7.1.6), UTP-dependent
10 pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2).

19. A foodstuff or food prepared from or comprising the invention according to any one of the preceding claims.

15

20. Use of any one or more of galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) as a selection means for selecting a genetically transformed cell over a non-transformed cell.

20

21. Use of any one or more of a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2) for providing a selection means for selecting a genetically transformed cell over a non-transformed
25 cell.

22. A prokaryote comprising a nucleotide sequence coding for galactokinase (EC 2.7.1.6), UTP-dependent pyrophosphorylase (EC 2.7.7.10), UDP-glucose-dependent uridylyltransferase (EC 2.7.7.12), UDP-galactose epimerase (EC 5.1.3.2); wherein the
30 nucleotide sequence comprises at least one intron which inactivates the nucleotide sequence or the expression product thereof in the prokaryote.

23. Use of any one or more of galactose, galactose-1-phosphate, UDP-galactose, or a derivative thereof, as a selection means for selecting a genetically transformed cell over a non-transformed cell.

5

24. A selection system that uses a component or a precursor therefor or a derivative thereof for selecting at least one genetically transformed cell from a population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but
10 wherein the component toxic to non-transformed cells, wherein the component and/or the derivative thereof and/or the precursor thereof is galactose or a derivative thereof or a precursor thereof.

25. A selection system that uses a component or a precursor therefor or a derivative
15 thereof for selecting at least one genetically transformed cell from a population of cells, wherein the at least one genetically transformed cell is transformed with a nucleotide sequence which encodes an expression product capable of utilising the component but wherein the component toxic to non-transformed cells.

20 26. A selection method for selecting from a population of cells one or more selectable genetically transformed cells,

wherein the population of cells comprises selectable genetically transformed cells and possible non-transformed cells;

25 wherein each of the selectable genetically transformed cells comprises a first expressable nucleotide sequence encoding a first expression product;

optionally wherein each of the selectable genetically transformed cells comprises an optional second expressable nucleotide sequence encoding a second expression product and/or an optional third expressable nucleotide sequence encoding a third expression product;

30 the method comprising the step of preparing the population of cells by transforming some or all of the cells in an initial population of cells containing non-

transformed cells with a heterologous nucleotide sequence so as to form the population of cells containing one or more selectable genetically transformed cells; and

selecting at least one of the selectable genetically transformed cells;

wherein the heterologous nucleotide sequence is any one or more of the first
5 expressable nucleotide sequence, the optional second expressable nucleotide sequence or the optional third expressable nucleotide sequence;

wherein each of the first expression product and the optional second expression product and the optional third expression product is independently selected from an enzyme capable of metabolising galactose or a derivative thereof or a precursor thereof.

10

27. A selection method or transformed cell, tissue, organ, or organism (or part thereof) substantially as described herein.

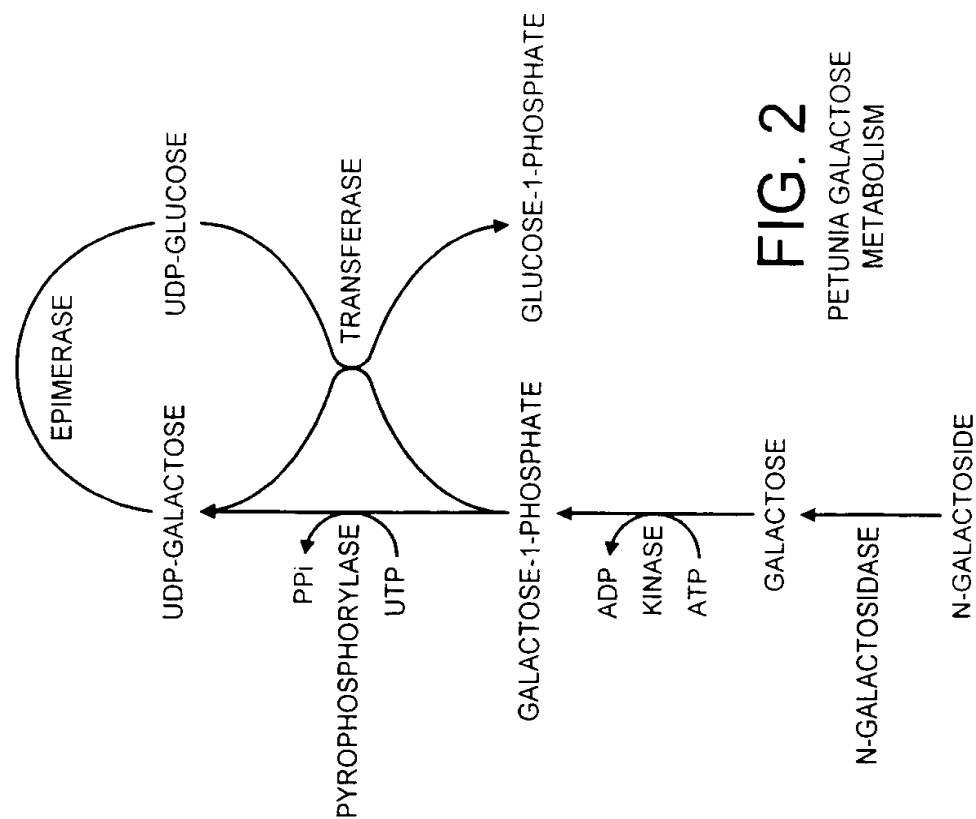


FIG. 2
PETUNIA GALACTOSE
METABOLISM

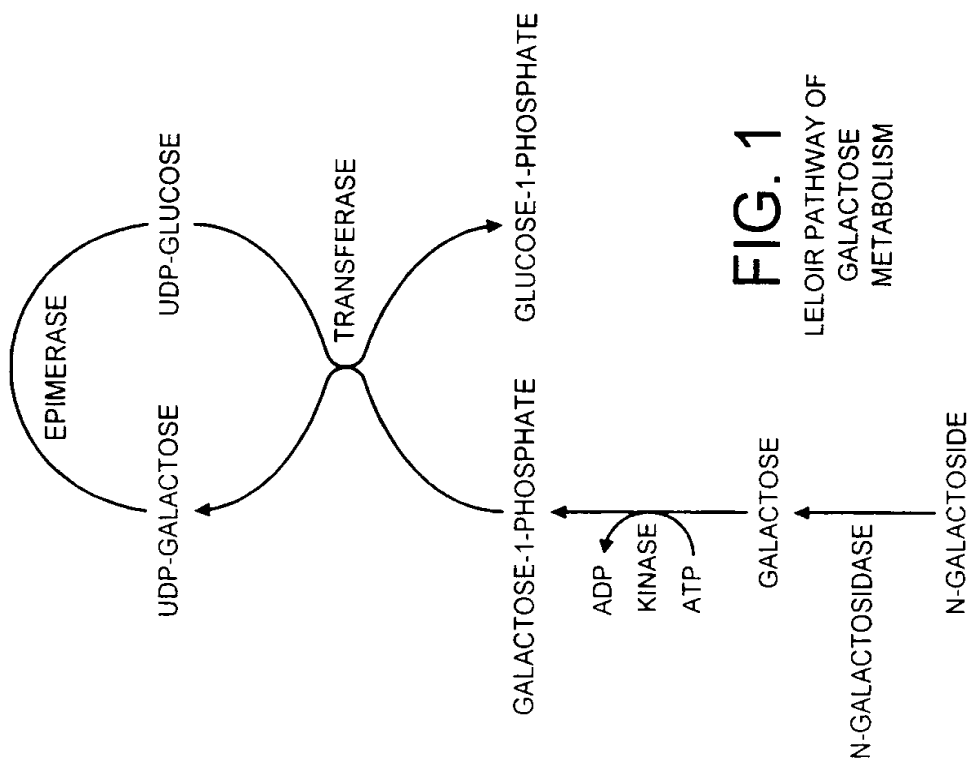


FIG. 1
LELOIR PATHWAY OF
GALACTOSE
METABOLISM

ACTGACGAACGACCATGACGCAATTTAATCCGGTTGATCATCCACATCGCCGCTACACCCGCTCACCGGGCAATGGATTCTGGTTTACCGGCACCGCG 100
 PstI M T Q F N P V D H P H R R Y N P L T G Q W I L V S P H R 28

CTAAGCGCCCTGGCAGGGGGCGCAGAAACGCCAGCCAAACAGGTGTACCTGGCAGCATCCAGATTGCTTCTCTGCGCAGGTAATGTGCGGGTGAC 200
 A K R P W Q G A Q E T P A K Q V L P A H D P D C F L C A G N V R V T 62

AGCGATAAAAACCCGATTACACCGGACTTACGTTTCTACTAATGACTTTGCGGCTTGTGCTGTGACACGCCAGATGCCCCAGAAAAGTCACGATCCG 300
 G D K N P D Y T G T Y V F T N D F A A L M S D T P D A P E S H D P 95

CTGATGCGTTGCCAGAGCGCGCGGCACAGCCGGGTGATCTGCTTTTACCGGATCACAGTAAACCGTGCCAGAGCTCAGCGTTGCAGCATTTGACGG 400
 L M R C Q S A R G T S R V I C F S P D H S K T L P E L S V A A L T 128

AAATCGTCAAAACCTGGCAGGAGCAAAACCGCGGAACCTGGGGAAAACGTACCCATGGGTGCAGGTTTTTGA AAAACAAAGCGCGCCGATGGGCTGCTCTAA 500
 E I V K T W Q E Q T A E L G K T Y P W V Q V F E N K G A P M G C S N 162

CCGCATCCGCACGGTCAGATTGGGCAATAGCTTCCTGCTAACAAGCTGAGCGGAAGACCGCTGCAAAAAGAAATATTTGCCGAACAGAAATCA 600
 P H P H G Q I W A N S F L P N E A E R E D R L Q K E Y F A E Q K S 195

CCAATGCTGGTGATTATGTTACGCGGAGCTGGCAGCGGTAGCCGTACCGTTGTGAAACCGAACACTGGTTAGCCGTGCTGCTTACTGGGCTGCCT 700
 P M L V D Y V Q R E L A D G S R T V V E T E H W L A V V P Y W A A 228

GGCGGTCGAAACGCTACTGCTGCCAAAGCCACGTTTACGGATCACCGATTGACCGACGCCCGCAGCGAGTCTGGCGCTGGCGTTGAAAAAGCT 800
 W P F E T L L L P K A H V L R I T D L T D A Q R S D L A L A L K K L 262

GACCAGTCGTTATGACAACTCTTCCAGTGCTCCTTCCGCTACTCTATGGGCTGGCAGCGCGGCCCATTTAATGGGAAGAGAATCAACACTGGCAGCTG 900
 T S R Y D N L F Q C S F P Y S M G W H G A P F N G E E N Q H W Q L 295

CACGCGCACTTTTATCCGCTCTGCTGGCTCCGCCACGTACGTAATTTATGGTTGTTATGAATGCTGGCAGAGACCCAGAGACCTGACCGCAG 1000
 H A H F Y P P L L R S A T V R K F M V G Y E M L A E T Q R D L T A 328

AACAGGCAGCAGCGTTTGGCGCAGTCAGCGATATCCATTTTCGCGAATCCGGAGTGAAGTCGACT 1069
 E Q A A E R L R A V S D I H F R E S G V - Sali 348

FIG. 3

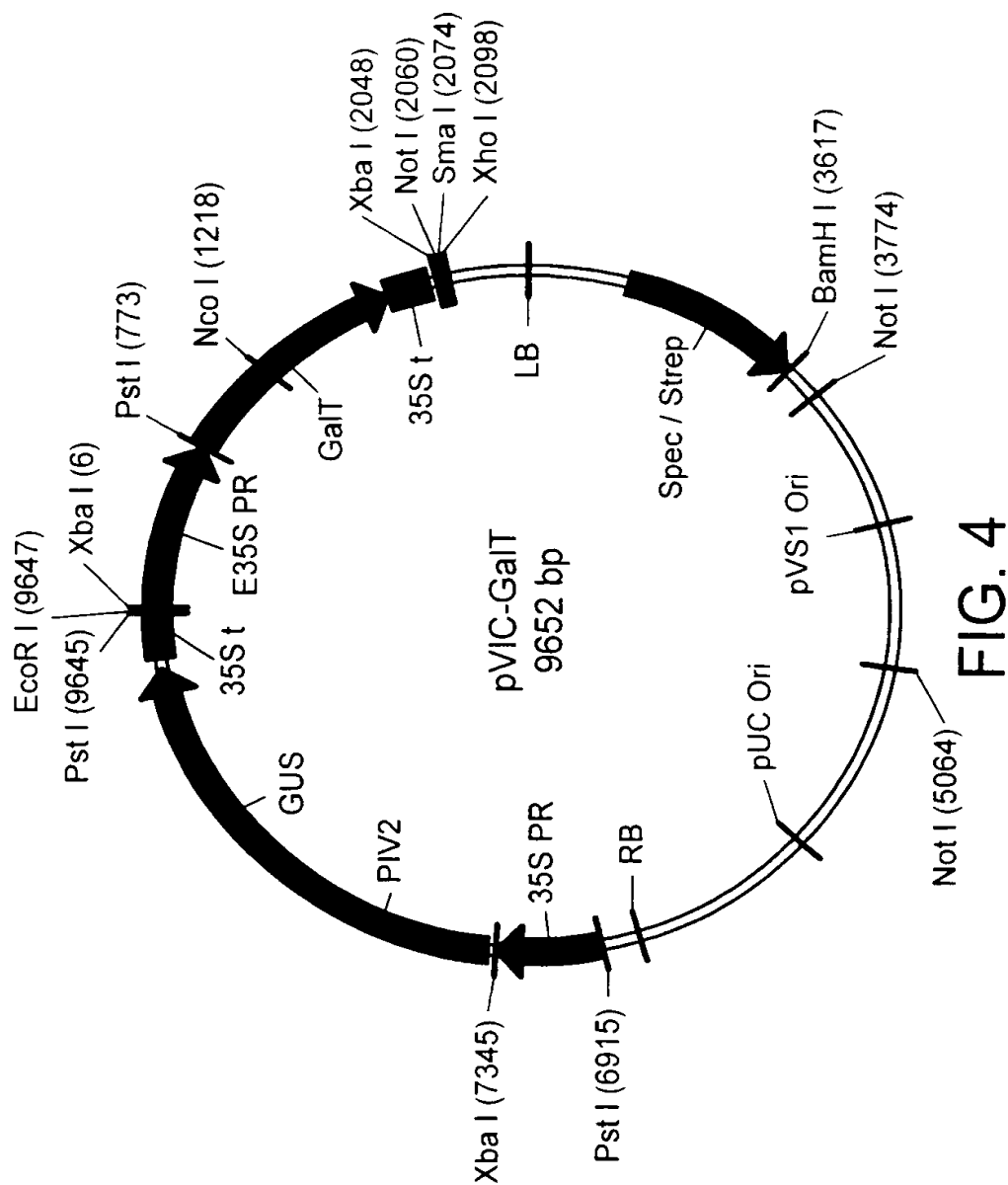


FIG. 4

A selection method for selecting from a population of cells one or more selectable genetically transformed cells is described. The population of cells comprises selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressible nucleotide sequence encoding a first expression product; and optionally a second expressible nucleotide sequence encoding a second expression product and/or a third expressible nucleotide sequence encoding a third expression product. A component is utilisable by the selectable genetically transformed cells by action of the first expressible nucleotide sequence or the first expression product and optionally by action of the optional second expressible nucleotide sequence or the optional second expression product and/or by action of the optional third expressible nucleotide sequence or the optional third expression product. The component can be present in an amount that is toxic to the non-transformed cells.

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INTERNATIONAL SEARCH REPORT

onal Application No

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/61 C12N15/82 C12N5/10 C12N1/21
A01H5/00

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Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Patent family members are listed in annex.

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28 January 2000

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Date of mailing (day/month/year) 06 April 2000 (06.04.00)	
International application No. PCT/IB99/01465	Applicant's or agent's file reference P005002WOCTH
International filing date (day/month/year) 11 August 1999 (11.08.99)	Priority date (day/month/year) 11 August 1998 (11.08.98)
Applicant JØRSBOE, Morten et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
14 February 2000 (14.02.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<p>The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland</p> <p>Facsimile No.: (41-22) 740.14.35</p>	<p>Authorized officer Olivia RANAIVOJAONA</p> <p>Telephone No.: (41-22) 338.83.38</p>
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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

2001

PCT

To:

D YOUNG & CO
Attn. HARDING, CHARLES
21 New Fetter Lane
London EC4A 1DA
UNITED KINGDOM

DAEL-14-4-00

*DS CTH
Records noted*

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference P005002WOCTH	Date of mailing (day/month/year) 14/02/2000
International application No. PCT/IB 99/01465	International filing date (day/month/year) 11/08/1999
Applicant DANISCO A/S et al.	

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
 The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland
 Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer Andria Overbeeke-Siepkens
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These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.



REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum)

P005002WOCTH

Box No. I	TITLE OF INVENTION	SELECTION METHOD
Box No. II APPLICANT		
Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)		
Danisco A/S Langebrogade 1 PO Box 17 DK-1001 Copenhagen K Denmark		
This person is also inventor.		
Telephone No.		
Facsimile No.		
Teleprinter No.		
State (i.e. country) of nationality: Denmark		
State (i.e. country) of residence: Denmark		
This person is applicant for the purposes of:		
all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box <input type="checkbox"/>		
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)		
Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)		
JØRSBOE, Morten Pandebjergvej 184 DK-4800 Nykøbing F Denmark		
This person is:		
applicant only <input type="checkbox"/>		
<input checked="" type="checkbox"/> applicant and inventor		
inventor only (if this check-box is marked, do not fill in below) <input type="checkbox"/>		
State (i.e. country) of nationality: Denmark		
State (i.e. country) of residence: Denmark		
This person is applicant for the purposes of:		
all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box <input type="checkbox"/>		
<input checked="" type="checkbox"/> Further applicant and/or (further) inventors are indicated on a continuation sheet		
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE		
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:		
<input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative		
Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)		
HARDING, Charles Thomas D Young & Co 21 New Fetter Lane London EC4A 1DA United Kingdom		
Telephone No. +44 23 8063 4816		
Facsimile No. +44 23 8022 4262		
Teleprinter No. 477667 YOUNGS G		
Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.		

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS***If none of the following sub-boxes is used, this sheet is not to be included in the request.***

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

BRUNSTEDT, Janne
Dueholm 18
DK-4000 Roskilde
Denmark

This person is:

applicant only

☒ applicant and inventor

inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

Denmark

State (that is, country) of residence:

Denmark

This person is applicant for the purposes of:

all designated States

all designated States except the United States of America

☒ the United States of America only

the States indicated in the Supplemental Box

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

JØRGENSEN, Kirsten
Guldborgvej 195
DK-4862 Guldborg
Denmark

This person is:

applicant only

☒ applicant and inventor

inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

Denmark

State (that is, country) of residence:

Denmark

This person is applicant for the purposes of:

all designated States

all designated States except the United States of America

☒ the United States of America only

the States indicated in the Supplemental Box

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

applicant only

applicant and inventor

inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

State (that is, country) of residence:

This person is applicant for the purposes of:

all designated States

all designated States except the United States of America

the United States of America only

the States indicated in the Supplemental Box

Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:

applicant only

applicant and inventor

inventor only (if this check-box is marked, do not fill in below)

State (that is, country) of nationality:

State (that is, country) of residence:

This person is applicant for the purposes of:

all designated States

all designated States except the United States of America

the United States of America only

the States indicated in the Supplemental Box

Further applicants and/or (further) inventors are indicated on a continuation sheet

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes, at least one must be marked):

Regional Patent

- ☒ **AP ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ **EA Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ **EP European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ **OA OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, please specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- | | |
|---|--|
| <input checked="" type="checkbox"/> AL Albania | <input checked="" type="checkbox"/> LS Lesotho |
| <input checked="" type="checkbox"/> AM Armenia | <input checked="" type="checkbox"/> LT Lithuania |
| <input checked="" type="checkbox"/> AT Austria | <input checked="" type="checkbox"/> LU Luxembourg |
| <input checked="" type="checkbox"/> AU Australia | <input checked="" type="checkbox"/> LV Latvia |
| <input checked="" type="checkbox"/> AZ Azerbaijan | <input checked="" type="checkbox"/> MD Republic of Moldova |
| <input checked="" type="checkbox"/> BA Bosnia and Herzegovina | <input checked="" type="checkbox"/> MG Madagascar |
| <input checked="" type="checkbox"/> BB Barbados | <input checked="" type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input checked="" type="checkbox"/> BG Bulgaria | <input checked="" type="checkbox"/> MN Mongolia |
| <input checked="" type="checkbox"/> BR Brazil | <input checked="" type="checkbox"/> MW Malawi |
| <input checked="" type="checkbox"/> BY Belarus | <input checked="" type="checkbox"/> MX Mexico |
| <input checked="" type="checkbox"/> CA Canada | <input checked="" type="checkbox"/> NO Norway |
| <input checked="" type="checkbox"/> CH AND LI Switzerland and Liechtenstein | <input checked="" type="checkbox"/> NZ New Zealand |
| <input checked="" type="checkbox"/> CN China | <input checked="" type="checkbox"/> PL Poland |
| <input checked="" type="checkbox"/> CU Cuba | <input checked="" type="checkbox"/> PT Portugal |
| <input checked="" type="checkbox"/> CZ Czech Republic | <input checked="" type="checkbox"/> RO Romania |
| <input checked="" type="checkbox"/> DE Germany | <input checked="" type="checkbox"/> RU Russian Federation |
| <input checked="" type="checkbox"/> DK Denmark | <input checked="" type="checkbox"/> SD Sudan |
| <input checked="" type="checkbox"/> EE Estonia | <input checked="" type="checkbox"/> SE Sweden |
| <input checked="" type="checkbox"/> ES Spain | <input checked="" type="checkbox"/> SG Singapore |
| <input checked="" type="checkbox"/> FI Finland | <input checked="" type="checkbox"/> SI Slovenia |
| <input checked="" type="checkbox"/> GB United Kingdom | <input checked="" type="checkbox"/> SK Slovakia |
| <input checked="" type="checkbox"/> GD Grenada | <input checked="" type="checkbox"/> SL Sierra Leone |
| <input checked="" type="checkbox"/> GE Georgia | <input checked="" type="checkbox"/> TJ Tajikistan |
| <input checked="" type="checkbox"/> GH Ghana | <input checked="" type="checkbox"/> TM Turkmenistan |
| <input checked="" type="checkbox"/> GM Gambia | <input checked="" type="checkbox"/> TR Turkey |
| <input checked="" type="checkbox"/> HR Croatia | <input checked="" type="checkbox"/> TT Trinidad and Tobago |
| <input checked="" type="checkbox"/> HU Hungary | <input checked="" type="checkbox"/> UA Ukraine |
| <input checked="" type="checkbox"/> ID Indonesia | <input checked="" type="checkbox"/> UG Uganda |
| <input checked="" type="checkbox"/> IL Israel | <input checked="" type="checkbox"/> US United States of America |
| <input checked="" type="checkbox"/> IN India | |
| <input checked="" type="checkbox"/> IS Iceland | |
| <input checked="" type="checkbox"/> JP Japan | <input checked="" type="checkbox"/> UZ Uzbekistan |
| <input checked="" type="checkbox"/> KE Kenya | <input checked="" type="checkbox"/> VN Viet Nam |
| <input checked="" type="checkbox"/> KG Kyrgyzstan | <input checked="" type="checkbox"/> YU Yugoslavia |
| <input checked="" type="checkbox"/> KP Democratic People's Republic of Korea | <input checked="" type="checkbox"/> ZW Zimbabwe |
| | Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after the issuance of this sheet: |
| <input checked="" type="checkbox"/> KR Republic of Korea | <input checked="" type="checkbox"/> AE United Arab Emirates |
| <input checked="" type="checkbox"/> KZ Kazakhstan | <input checked="" type="checkbox"/> ZA South Africa |
| <input checked="" type="checkbox"/> LC Saint Lucia | <input checked="" type="checkbox"/> CR Costa Rica |
| <input checked="" type="checkbox"/> LK Sri Lanka | <input checked="" type="checkbox"/> DM Dominica |
| <input checked="" type="checkbox"/> LR Liberia | |

Cautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the end of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Supplemental Box If the Supplemental Box is not used, this sheet need not be included in the request.

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

(a) in Part I:

(i) if more than two countries are designated applicants and/or inventors, and no communication is required, available;

(ii) if, in Box No. II, or in any of the side boxes of Box No. III, the information "Inventor" is indicated, in the Supplemental Box in the following:

(iii) if, in Box No. II, or in any of the side boxes of Box No. III, the inventor or the inventor-applicant is not an inventor for the purposes of all designated States or for the purposes of the United States of America;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents;

(v) if, in Box No. V, the name of any State or OAPI is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part";

(vi) if there are more than three earlier applications whose priority is claimed;

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty;

In such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

In such case, write "Continuation of Box No. III" and indicate for each additional box the same type of information as required in Box No. III. The number of the additional Box is the applicant's State or country. The information of the first additional Box is:

In such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Box No. II and No. III" (as the case may be); indicate the name of the applicant(s) involved and, next to each such name, the State(s) and/or, where applicable, ARIPO, Eureka, European or OAPI patent for the purposes at which the named person is applicant;

In such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be); indicate the name of the inventor(s) and, next to each such name, the State(s) and/or, where applicable, ARIPO, Eureka, European or OAPI patent for the purposes at which the named person is inventor;

In such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Part No. IV;

In such case, write "Continuation of Box No. V" and indicate for each State involved or OAPI, and after the name of each of the State(s) or OAPIs, the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

In such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;

In such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below;

Continuation of Box No IV

PURVIS, William Michael Cameron
COTTER, Ivan John
PILCH, Adam John Michael
CRISP, David Norman
ROBINSON, Nigel Alexander Julian
HARRIS, Ian Richard
HARDING, Charles Thomas
TURNER, James Arthur
MALLALIEU, Catherine Louise
PRICE, Paul Anthony King
PRATT, Richard Wilson
HOLMES, Miles Keeton
HORNER, David Richard
MASCHIO, Antonio
NACHSHEN, Neil Jacob
POTTER, Julian Mark

Box No. VI PRIORITY CLAIM

Further priority claims are indicated in the Supplemental Box

The priority of the following earlier application(s) is hereby claimed:

	Filing Date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
			national application: country	regional application: regional Office	international application: receiving Office
item (1)	11 Aug 1998 (11/8/1998) 11 August 1998	9817465.9	[UK] G-B		
item (2)					
item (3)					

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): item (1)

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA)
(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA / EPO

Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority):

Date (day/month/year)

Number:

Country (or regional Office):

Box No. VII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request	5
description (excluding sequence listing part)	73
claims	8
abstract	1
drawings	3
sequence listing part of description	
Total number of sheets	90

This international application is accompanied by the item(s) marked below:

1. ☐ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority documents(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other (specify): Letter

Figure of the drawings which should accompany the abstract:

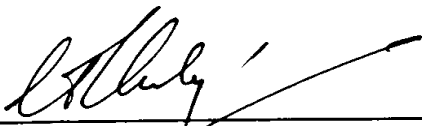
Language of filing of the international application:

English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request)

C T Harding



For receiving Office use only

1. Date of actual receipt of the purported international application:

11 August 1999 (11.08.99)

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

2. Drawings:

☐ received:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

☐ not received:

5. International Searching Authority specified by the applicant:

ISA /

EP

6. ☒ Transmittal of search copy delayed until search fee paid

Date of receipt of the record copy by the International Bureau:

For International Bureau use only

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

HARDING Charles Thomas
D YOUNG & CO
21 New Fetter Lane
London EC41 1DA
GRANDE BRETAGNE

DS

CTH

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

CTH

Date of mailing
(day/month/year)

20. 10. 00

Applicant's or agent's file reference
P005002WOCTH

IMPORTANT NOTIFICATION

International application No.
PCT/IB99/01465

International filing date (day/month/year)
11/08/1999

Priority date (day/month/year)
11/08/1998

Applicant
DANISCO A/S et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
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Authorized officer

Vullo, C

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PCT

Application No.:

PCT/IB99/01465

Note on an informal communication by telephone with the Applicant

A copy of this note is being sent to the Applicant for information

To Support Service: return the file to 1st examiner !

Participants

Agent: L. Holliday

Examiner(s): Kurz, B

Summary of the communication

Information about formal and substantive matters:

1. The representative was asked to file the amendments of 2.8.2000 (letter dated 1.8.2000) in retyped form.
2. The representative was informed that a negative IPER will be issued.

16/10/2000

.....
Date (day / month / year)

3. kurz

.....
Kurz, B
Authorized officer of IPEA

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference P005002WOCTH	<div style="display: flex; justify-content: space-between;"> <div>FOR FURTHER ACTION</div> <div>See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)</div> </div>	
International application No. PCT/IB99/01465	International filing date (<i>day/month/year</i>) 11/08/1999	Priority date (<i>day/month/year</i>) 11/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant DANISCO A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 9 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☒ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 14/02/2000	Date of completion of this report 23.10.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Kurz, B Telephone No. +49 89 2399 7319

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/IB99/01465

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-46,48-73	as originally filed	
47	with telefax of	02/08/2000

Claims, No.:

1-24	with telefax of	02/08/2000
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Drawings, sheets:

1/3-3/3	as originally filed
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2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
- ☐ copy of the earlier application whose priority has been claimed.
 - ☐ translation of the earlier application whose priority has been claimed.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IB99/01465

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	9, 18, 21
	No:	Claims	1-8, 10-17, 19, 20, 22-24
Inventive step (IS)	Yes:	Claims	-
	No:	Claims	1-24
Industrial applicability (IA)	Yes:	Claims	1-24
	No:	Claims	-

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB99/01465

Re Item II

Priority

Although Figures 3 and 4 of the present application were not part of the priority document, their subject-matter formed part of the written disclosure of the priority document. Therefore Figures 3 and 4 of the present application are covered by the priority rights.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

- D1: SCHÜMPERLI, D., ET AL.: 'Efficient expression of Escherichia coli galactokinase gene in mammalian cells' PROC. NATL. ACAD. SCI., vol. 79, 1982, pages 257-261
- D2: WO 93 05163 A (DANISCO) 18 March 1993 (1993-03-18)
- D3: DÖRMANN ET AL: 'The role of UDP-glucose epimerase in carbohydrate metabolism of Arabidopsis' PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 13, no. 5, 1 March 1998 (1998-03-01), pages 641-652
- D4: WO 94 20627 A (SANDOZ AG ;SANDOZ AG (DE); SANDOZ LTD (CH); BOJSEN KIRSTEN (DK); D) 15 September 1994 (1994-09-15)
- D5: EP-A-0 235 112 (SMITHKLINE BECKMAN CORP) 2 September 1987 (1987-09-02)
- D6: DÖRMANN ET AL: 'Functional expression of Uridine 5'diphospho-glucose 4-epimerase (EC 5.1.3.2) from Arabidopsis thaliana in Saccharomyces cerevisiae and Escherichia coli' ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, US, NEW YORK, US, XP002076759 ISSN: 0003-9861
- D7: JOERSBO, M., ET AL.: 'Analysis of mannose selection used for transformation of sugar beet' MOLECULAR BREEDNG, vol. 4, April 1998 (1998-04), pages 111-117
- D8: WO 96 31612 A (SEMINIS VEGETABLES ;TRULSON ANNA JULIA (US); GREEN CHARLES EDWARD) 10 October 1996 (1996-10-10)

D9: JOHANSEN I E: 'INTRON INSERTION FACILITATES AMPLIFICATION OF CLONED VIRUS CDNA IN ESCHERICHIA COLI WHILE BIOLOGICAL ACTIVITY IS REESTABLISHED AFTER TRANSCRIPTION IN VIVO' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, no. 22, 29 October 1996 (1996-10-29), pages 12400-12405

The present application deals with a selection method using the ability of plants to grow on D-galactose as a carbon source as a distinguishing feature.

1. Novelty (Article 33(2) PCT):

The following claims are considered not to be novel:

Claims 1-8, 10-17, 19, 20, 22-24

1.1 Document D1 discloses the insertion of the E. coli galactokinase gene into a plasmid. The plasmid is then used for the transfection of hamster cells deficient in galactokinase activity. The complementation of the defect leads to the selective survival of the transformed cells which selectively grow on galactose as sole carbon source in the medium.

According to the present wording claim 24 is therefore anticipated by D1.

1.2 Document D2 discloses a selection method making use of the introduction of a nucleotide sequence (or a co-introduced nucleotide sequence), wherein the product of the introduced sequence induces or increases the positive effect of a compound or nutrient. Of particular relevance are claims 1-7, 21 and 24 of D2 in which the use of galactose as a compound and of UDP-galactose epimerase as an enzyme useful for the selection of transformed plant cells are explicitly mentioned. As acknowledged by the applicant on page 22, l. 30 and 31 of the present application, the toxicity of galactose for most plants was known for decades. Although not explicitly mentioned in D2, a general adverse effect of galactose thus can be assumed also for the method of D2. Consequently, the method of D2 exhibits positive and negative effects (see p. 12, l. 10 ff of D2). In view of D2 claims 1-8, 10-17, 19, 20, 23 and 24 cannot be considered novel.

- 1.3 Documents D3 to D8 are relevant for novelty and inventive step of various claims of the present application. These documents represent the state of the art concerning the enzymes of galactose metabolism, especially in plants, and the use of sugars such as mannose or xylose as selective markers. However, their subject-matter does not contribute to any objections going beyond those already mentioned in 1.1 and 1.2. For the moment, D3 to D8 therefore will not be discussed in detail.
- 1.4 The documents cited under VI are not referred to in detail as all the claims which are anticipated by these documents are as well anticipated by documents published before the priority date of the present application (see 1.1-1.3).

2. Inventive step (Article 33(3) PCT):

Claims 9, 18 and 21 which were not objected to under Article 33(2) PCT are considered not to involve any inventive activity as required by Article 33(3) PCT.

- 2.1 Although the documents cited under V, 1. disclose various ways of selecting transformed plant cells without using antibiotics or herbicide resistance genes as selective markers, none of them directly mentions a kit comprising the respective construct and the medium. However, it is clear that both are necessary to carry out the selection procedure. As a consequence, claim 9 of the present application does not involve any inventive activity. In contrast, it might also be concluded that the features are implicitly comprised in the prior art and therefore not novel.
- 2.2 Claim 18 relates to the use of the enzymes galaktokinase, UTP-dependent pyrophosphorylase, and UDP-glucose dependent uridylyltransferase. The pathway by which galactose is metabolized in plants and other organisms is known and thus also the enzymes that may contribute to the necessary reactions (see D2, Figure 7). Document D5 discloses the gal operon of *Streptomyces* comprising the galE, galT and galK genes. D5 also proposes (page 18) the use of these genes of the gal operon as a selection marker in a host which is unable to utilise galactose because it contains a defect within one of the respective genes. In view of D5 the expression of these genes in plants not able to utilise galactose cannot be considered inventive.

- 2.3 Claim 21 relates to the use of a prokaryote comprising a nucleic acid which is prevented from being expressed by the introduction of at least one intron. Document D9 discloses that the introduction of an intron facilitates the expression of cloned cDNA in *E. coli*. D9 generally teaches that intron insertion facilitates manipulation and amplification of cloned DNA fragments (see abstract). The introduction of an intron in a gene involved in the galactose metabolism can therefore not be considered inventive.
- In addition, at the time the present application was filed, several human genes involved in the galactose metabolism were already cloned. The genomic sequences of these genes contain introns and therefore might have been used for the purpose indicated without further alteration.
- 2.4 In case that novelty of some of the claims discussed in V, 1. should be restored, the subject-matter of claims 1-8, 10-17, 19, 20, and 22-24 will be considered not inventive in view of documents D2 or D4 in combination with D3.
- Documents D2 and D4 both disclose selection methods for transformed plant cells. In D2, galactose and UDP-galactose epimerase are used, and in D4 mannose or xylose are used in combination with mannose-6-phosphate isomerase or xylose isomerase, respectively.
- Document D3 teaches (p. 642, left column), that mannose as well as galactose are toxic to plants. On p. 650 it is stated that UDP-glucose epimerase is an interesting transformation marker for genetic engineering of crop plants.
- At the priority date of the present application it was therefore known how to use enzymes involved in the carbohydrate metabolism as a selective marker and it was also known which compounds are generally useful. The detailed provision of one of these possibilities which were already under discussion does not represent any inventive activity. Furthermore, also the enzymes involved in the galactose and glucose biosynthetic pathways were known (e.g. see D2, Figure 7).

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB99/01465

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date (day/month/year)	Filing date (day/month/year)	Priority date (valid claim) (day/month/year)
PCT/GB98/00367	13.08.98	05.02.98	07.02.97&02.02.98
PCT/CA99/00056	29.07.99	22.01.99	22.01.98& 23.01.98
PCT/IB98/00886	03.12.98	27.05.98	28.05.97

The subject-matter of the P-documents cited in the search report is related to the subject-matter of claims 4-19 and 27 of the present application.

Re Item VIII

Certain observations on the international application

The application lacks clarity with respect to the following:

1. In claims 1-4, 6, and 22-24 reference is made to a "component and/or a precursor thereof" and to galactose "or a precursor thereof" or "a derivative" thereof. However, it is not clear which substances fall within the scope of the claims (Article 6 PCT). At present a skilled person is not able to decide whether a certain substance falls within the scope of the claims or not.
In case that the component is galactose, it is not clear which criteria must be met by a molecule to fall under the definition "derivative". It is also not clear what criteria a precursor of galactose must meet. In the broadest sense, all sugars (e.g. also disaccharides) can be seen as precursors as long as they can be transformed into galactose somehow. In this case the wording is not only unclear but the subject-matter is insufficiently disclosed (Articles 5 and 6 PCT).
The subject-matter is even more obscure in case that the substance present in the medium is a precursor. It is not clear what finally results from this precursor and which substance finally will be utilised by the cells.
With the present wording neither the enzyme nor the substrate are specified.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IB99/01465

2. General statements in the description which imply that the extent of protection may be expanded in some vague and not precisely defined way, such as the "spirit" of an invention (p. 71, l. 19/20 of the application), are objected to (Article 6 PCT and PCT Gazette Section IV, III-4.3a).

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference P005002WOCTH	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/IB 99/ 01465	International filing date (day/month/year) 11/08/1999	(Earliest) Priority Date (day/month/year) 11/08/1998
Applicant DANISCO A/S et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☒ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☐ the text is approved as submitted by the applicant.

☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☒ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention

3

☐ None of the figures.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

A selection method for selecting from a population of cells one or more selectable genetically transformed cells is described. The population of cells comprises selectable genetically transformed cells and possible non-transformed cells. Each of the selectable genetically transformed cells comprises a first expressible nucleotide sequence encoding a first expression product; and optionally a second expressible nucleotide sequence encoding a second expression product and/or a third expressible nucleotide sequence encoding a third expression product.

A component is utilisable by the selectable genetically transformed cells by action of the first expressible nucleotide sequence or the first expression product and optionally by action of the optional second expressible nucleotide sequence or the optional second expression product and/or by action of the optional third expressible nucleotide sequence or the optional third expression product. The component can be present in an amount that is toxic to the non-transformed cells.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01465

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N15/61 C12N15/82 C12N5/10 C12N1/21
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHÜPERELI, D., ET AL.: "Efficient expression of Escherichia coli galactokinase gene in mammalian cells" PROC. NATL. ACAD. SCI., vol. 79, 1982, pages 257-261, XP002129147 the whole document ---	1-10, 14, 20, 21, 23, 24, 26, 27
X	AHMED, A., ET AL.: "Plasmid vectors for positive galactose-resistance selection of cloned DNA in Escherichia coli" GENE, vol. 28, 1984, pages 37-43, XP002129148 page 41, left-hand column --- -/--	4, 20, 21, 23

☒ Further documents are listed in the continuation of box C☒ Patent family members are listed in annex

Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

28 January 2000

Date of mailing of the international search report

14/02/2000

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Maddox, A

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01465

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	DATABASE CAB 'Online! CAB INTERNATIONAL, WALLINGFORD, OXON, GB AN 84:135604, HESS, D., ET AL.: "Bacterial transferase activity expressed in Petunia protoplasts" XP002129152 abstract & JOURNAL OF PLANT PHYSIOLOGY, vol. 116, no. 4, 1984, pages 261-272, ---	16,18
X	WO 93 05163 A (DANISCO) 18 March 1993 (1993-03-18) ---	2-21,23, 26,27
Y	page 11, line 12 - line 14; claim 24 ---	1,18,24
X	DÖRMANN ET AL: "The role of UDP-glucose epimerase in carbohydrate metabolism of Arabidopsis" PLANT JOURNAL, GB, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 13, no. 5, 1 March 1998 (1998-03-01), pages 641-652, XP002082772 ISSN: 0960-7412 ---	4-12,16
Y	see whole document particularly page 649 right col. last para. - page 650 line 2 ---	1,18,24
X	WO 94 20627 A (SANDOZ AG ;SANDOZ AG (DE); SANDOZ LTD (CH); BOJSEN KIRSTEN (DK); D) 15 September 1994 (1994-09-15) ---	25
A	the whole document ---	1,24
X	EP 0 235 112 A (SMITHKLINE BECKMAN CORP) 2 September 1987 (1987-09-02) page 12, line 32 -page 14, line 3 page 18, line 3 -page 19, line 7 ---	2-10,20, 21,23
X	DÖRMANN ET AL: "Functional expression of Uridine 5'diphospho-glucose 4-epimerase (EC 5.1.3.2) from Arabidopsis thaliana in Saccharomyces cerevisiae and Escherichia coli" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, US, NEW YORK, US, XP002076759 ISSN: 0003-9861 page 29, right-hand column, paragraph 2 ---	4-10,20, 21,23
X	WO 96 09374 A (SMITHKLINE BEECHAM CORP ;UNIV PENNSYLVANIA (US)) 28 March 1996 (1996-03-28) page 21, line 28 -page 24 ---	22
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01465

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	DAUDE, N., ET AL.: "Homo sapiens UDP-galactose 4' epimerase (GALE) gene, complete cds." EMBL ACCESSION NO:AF022382, 16 March 1998 (1998-03-16), XP002129149 the whole document ---	22
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN 1993:4448, LESLIE, N.D., ET AL.: "The human galactose-1-phosphate uridylyltransferase gene" XP002129153 abstract & GENOMICS, vol. 14, no. 2, 1992, pages 474-480, ---	22
X	JOERSBO, M., ET AL.: "Analysis of mannose selection used for transformation of sugar beet" MOLECULAR BREEDING, vol. 4, April 1998 (1998-04), pages 111-117, XP002129150 the whole document ---	25
X	WO 96 31612 A (SEMINIS VEGETABLES ;TRULSON ANNA JULIA (US); GREEN CHARLES EDWARD) 10 October 1996 (1996-10-10) the whole document ---	25
P,X	WO 98 35047 A (DONALDSON IAIN ALASDAIR ;DANISCO (DK); BOJSEN KIRSTEN (DK); JOERSB) 13 August 1998 (1998-08-13) the whole document ---	25
P,X	WO 99 37786 A (DONG JIN ZHUO ;GEORGES FAWZY (CA); DATLA RAJU S S (CA); CANADA NAT) 29 July 1999 (1999-07-29) example 19 ---	4-12,18
P,X	WO 98 54334 A (PETERSEN STEEN GULDAGER ;DANISCO (DK); BRUNSTEDT JANNE (DK); JOERS) 3 December 1998 (1998-12-03) the whole document ---	4-12,18, 20,21,23

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01465

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
A	<p>DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US AN 75:75196, ROBERTS, R.M., ET AL.: "Growth inhibition and metabolite pool levels in plant tissues fed D-glucosamine and D-galactose" XP002129154 abstract & PLANT PHYSIOL., vol. 48, no. 1, 1971, pages 36-45,</p> <p>---</p>	1-27
A	<p>HALDRUP, A., ET AL.: "The xylose isomerase gene from Thermoanaerobacterium thermosulfurogenes allows effective selection of transgenic plant cells using D-xylose as the selection agent" PLANT MOLECULAR BIOLOGY, vol. 37, May 1998 (1998-05), pages 287-296, XP002129151 the whole document</p> <p>---</p>	1-27
A	<p>JOHANSEN I E: "INTRON INSERTION FACILITATES AMPLIFICATION OF CLONED VIRUS CDNA IN ESCHERICHIA COLI WHILE BIOLOGICAL ACTIVITY IS REESTABLISHED AFTER TRANSCRIPTION IN VIVO" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, no. 22, 29 October 1996 (1996-10-29), pages 12400-12405, XP002069935 ISSN: 0027-8424 the whole document</p> <p>-----</p>	22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 99/01465

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9305163	A	18-03-1993	AT 181963 T	15-07-1999
			AU 664200 B	09-11-1995
			AU 2556792 A	05-04-1993
			CA 2110401 A	18-03-1993
			CZ 284828 B	17-03-1999
			CZ 9400444 A	15-06-1994
			DE 69229556 D	12-08-1999
			DE 69229556 T	11-11-1999
			EP 0530129 A	03-03-1993
			EP 0601092 A	15-06-1994
			EP 0896063 A	10-02-1999
			HU 69606 A	28-09-1995
			JP 6511146 T	15-12-1994
			NZ 244135 A	27-04-1995
			SK 22294 A	06-07-1994
			US 5994629 A	30-11-1999
WO 9420627	A	15-09-1994	AU 682495 B	09-10-1997
			AU 6207794 A	26-09-1994
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